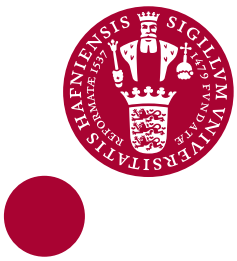

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role of parity, dry period and nutrient supply

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Publication date:
2009

Document version
Publisher's PDF, also known as Version of record

DEPARTMENT OF BASIC ANIMAL AND VETERINARY SCIENCES
FACULTY OF LIFE SCIENCES
UNIVERSITY OF COPENHAGEN

Mammary remodelling and metabolic activity in dairy goats: Role of parity, dry period and nutrient supply

PhD thesis

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Submitted: 16 November 2009

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Mammary remodelling and metabolic activity in dairy goats: Role of parity, dry period and nutrient supply
PhD Thesis 2009©Sina Safayi
ISBN 978-87-7611-332-2
Printed by Samfundslitteratur Grafik, Frederiksberg, Denmark

Preface

This dissertation is to fulfil the requirements for obtaining a PhD degree from Department of Basic Animal and Veterinary Sciences, Faculty of Life Sciences, University of Copenhagen, Denmark. This PhD project has been co-financed by the Danish Research Council (FTP; 2006-2009) and by private source (Mrs. Maryam Naminan; 2004-2006). All the animal experiments included in this thesis have been carried out at experimental facilities at Faculty of Life Sciences, University of Copenhagen (2004-2008).

The whole PhD programme has been done under the supervision of Associated Professor Dr. Mette Olaf Nielsen, to whom I am very thankful for trusting in me and giving me the option to be her student, and for her great advices, patience, and generosity not only as a supervisor, but also as a valuable friend.

My appreciation goes to Professor Chris. H. Knight, Research Scientist Marion Boutinaud and Professor Kristina Dahlborn for accepting to be members of the assessment committee for my thesis defence and for their time and efforts.

Part of the PCR work was performed at the Faculty of Agricultural Sciences, Aarhus University (2006) under supervision of Senior Scientists Dr. Peter Kappel Theil and Dr. Kristen Sejrsen, to whom I am very thankful for their valuable advices and collaborations. Furthermore, I had the pleasure of working with Professor R.J. (Bob) Collier and Research Specialist Sr. Jayne Collier, Department of Animal Sciences, University of Arizona, AZ, USA, to whom I would like to express my appreciation for their great hospitality. There (2009), as a visiting scholar, I was involved in a project on "Role of Prostaglandin E2 (PGE2) on Growth and Differentiation of Bovine Mammary Epithelial Cells" to learn the techniques related to cell culture.

I would also like to thank for the valuable advices I got from Drs. V.S. Elbrønd (histology), C.Th. Ekstrøm (statistics), and D.L. Wulfsohn (stereology) at University of Copenhagen.

It was an honour to co-supervise two MSc theses projects which were undertaken by my friends Lei Hou (Holly) and Marie Engbæk at the Faculty of Life Sciences, University of Copenhagen (2007-2008).

I would like to acknowledge many of my colleagues at both University of Copenhagen and Aarhus University, especially Vibeke G. Chrsitensen, Dennis S. Jensen, Ruth Jensen, Malin P. Tygesen, Jan V. Nørgaard, Sanne Husted, Marina Kjærgaard, Martin P. Jensen, Anna H. Kongsted, Sophia G. Moesgaard, Kasper B. Poulsen, Helle A.V. Rubby, Birgitte Holle, Betina Dahl, Kristine F. Dalbach, Alice N. Rasmussen, Hanne L. P. Carlsson, Christina T. Kjempff, Zaida R. Rasmussen, Anne Friis Petersen, Luminita Zamfirescu, Lise Kolbøl, Lene A. Agersted, Marie-Louise Rosenlund, Karin Kold, Mario Acquarone, Alishir Kiani, Lotte B. Strøbech, Merethe N. Stubgaard, Vibeke Dantzer, Allan Danfær, Ebba de N. Harrison, Inge Mejdahl, Jan H. Woller, Vibeke B. Hansen and Lennart E. Carlsen for their valuable assistance, advices, inspiration, and the share they have in my PhD thesis.

I would also like to express my appreciation to my x-wife (Gulli) and her family for the years I had their support.

Last but not the least, I would like to thank and dedicate this thesis to my beloved mother (Maryam) and sister (Saba) for their never ending love and support.

Sina Safayi,
November 2009

List of Abbreviations

18S RNR1	18S Ribosomal RNA I
A	Arterial concentrations
AAT	Amino acids absorbed from the small intestine
ACE	Sodium acetate
ACTB	Beta-Actin
ANGPT	Angiopoietin
AVD	Arterial-milk vein concentration difference
BAX	Bcl2-Associated X Protein
BCL2	B-Cell CLL/Lymphoma 2
BDP	Before dry period
BHB	Beta-hydroxy-butyrate
CA4	Carbonic Anhydrase IV
CCND1	Cyclin D1
CL	Continuous lactation
COX	Cyclooxygenase
DM	Dry matter
E	Extraction
EAA	Essential amino acids
ECM	Energy corrected milk yield
EDP	Early dry period
EL	Early lactation
Flk1	Fetal liver kinase 1
Flt1	fms-like tyrosine kinase
GAPDH	GlycerAldehyde-3-Phosphate Dehydrogenase
GLU	Glucose
H&E	Haematoxylin-Eosin
HKG	Housekeeping genes
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
Ki-67	Ki-67 antigen localization
LALBA	Alpha-Lactalbumin
LCFA	Long-chain fatty acids
LDP	Late dry period
LL	Late lactation
LPL	Lipoprotein lipase
LPT	Leptin
LPTR	Leptin Receptor
LTF	Lactoferrin (Lactotransferrin)
MBF	Mammary blood flow
MDP	Mid dry period
MEC	Mammary Epithelial Cell
MP	Multiparous
NEAA	Nonessential amino acids
NEFA	Non-esterified fatty acids
NL	Normal lactation
P	Parity
PAS	Periodic Acid-Schiff
PBV	Protein balance in rumen
PP	Primiparous
PRLR	Prolactin Receptor
PTGIS	Prostocylcine Synthase
RTK	Tyrosine Kinase Tie2 Receptor
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RQ	Respiratory quote
S	Stage of lactation
SAL	Saline
SEM	Standard error of mean
SFU	Scandinavian Feed Unit
T	Treatment
TBXAS	Thromboxane A2 Synthase
TCO_2	Total CO_2
TG	Triglycerol
TGFB	Transforming Growth Factor Beta
TGFB1R	Transforming Growth Factor Beta I Receptor
TO_2	Total O_2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

List of Contents

Summary

Milk production is generally lower, but lactation persistency higher in primiparous (**PP**) than multiparous (**MP**) ruminants. This may be related to differences in development and maintenance of mammary gland function, but the underlying mechanisms are not well understood. Furthermore, milk production in MP ruminants is determined not only by the genetic capacity of the animal but also by management factors such as duration of the dry period in between successive lactations. The dry period appears to be essential for MP dairy animals to ensure that the mammary epithelial cell (**MEC**) population can be effectively renewed as one lactation comes to an end and prior to onset of the next lactation. Generally, the level of milk production and the changes in milk yield over the course of lactation depend on three main factors: 1) the number of MEC, which in turn is affected by the balance between the rate of epithelial cell proliferation and apoptosis; 2) the secretory activity of these cells, which in turn is affected by their differentiation; and 3) the provision of nutrients and removal of metabolic waste products via the blood. The present thesis aimed to address the hypotheses that 1) differences between PP and MP animals with respect to milk production and lactation persistency may be related to differences in mammary growth and remodelling also during lactation, 2) the factors responsible for interfering with mammary remodelling in continuous lactation throughout the dry period into the subsequent lactation, are the same factors involved also in determination of lactation persistency and performance differences between PP and MP animals, 3) milk protein synthesis in the lactating mammary gland will be less sensitive towards variations in nutrient supply in late compared to early lactation, 4) minor deficiencies in dietary provision of protein can be compensated by provision of energy (ATP) yielding substrates to sustain milk (protein) synthesis.

The hypotheses were addressed in four papers based on three experiments with dairy goats. In Experiments 1 and 2, mammary remodelling was compared in PP and MP goats during lactation. In multiparous goats, the importance of the dry period for mammary remodelling was further assessed by unilateral drying off of one gland appr. 9 weeks prior to parturition followed by a normal lactation (**NL**), whilst omitting the dry period and thus subjecting the other gland to a continuous lactation (**CL**). In Experiment 3, the importance of nutrient supply in regulation of milk (protein) synthesis was assessed. This was done by providing extra nutrients through intravascular isoosmotic infusion of nutrients (essential amino acids, acetate or glucose) and determining the impact on milk yield and mammary nutrient uptake. The

goats were fed a basal diet providing appr. 90 and 80% of requirements for net energy and amino acids absorbable from the small intestine, respectively. This was done in early (**EL**) and late lactation (**LL**) to assess the changes in mammary sensitivity towards variations in nutrient supply with advancing lactation, and hence to evaluate whether there might be a physiological foundation for development of graded protein recommendations for ruminants. Mammary biopsies were obtained in Experiments 1 and 2 from both mammary glands and blood samples were obtained in all experiments for determination of arterio-venous concentration differences across each of the mammary glands at different stages of gestation-lactation. Gene transcription relating to MEC turnover, lactogenesis, angiogenesis and vascular function was quantified by real time RT-PCR, mammary morphology characterized (quantitative histology), and cell turnover determined (TUNEL and Ki-67). Whole blood was analysed for acid-base parameters and plasma for contents of key metabolites and hormones.

The experimental results demonstrated that initiation of lactation in the PP mammary gland is associated with development and growth of the gland in early lactation, which continues for a longer period of time compared to MP glands. Evidence has been provided that the set of genes regulating the orchestrated changes in mammary epithelial cell and vascular function during lactation, are also basically the same set of genes explaining different patterns of development between PP and MP goats. Both vascular and mammary epithelial cell development are thus determinants of lactation performance and can explain the reported higher lactation persistency in PP goats compared to MP. But the underlying reason why mammary development in PP animals have a different trajectory compared to MP remains to be established. No specific genomic factor(s) could account for the differences in mammary (re)development and MEC survival between PP and MP glands.

We confirmed that omitting the dry period is possible although with some difficulty in goats, and without major impact on milk yield in the subsequent lactation, which is in contrast to the cow. MEC renewal was suppressed in CL glands, which resulted in a smaller MEC population in the subsequent lactation. At the time of parturition (and throughout lactation), the mammary glands subjected to CL had smaller alveoli, more fully differentiated MEC and a substantially larger capillary fraction compared with NL glands, and CL gland thus resembled a normally lactating gland in a more advanced stage of lactation. None of the studied genomic factors could account for the differences in mammary (re)development and MEC survival between NL and CL glands. Efficiency of mammary nutrient extraction was unaffected by CL, and the more MECs in a more fully differentiated state are therefore not

necessarily more metabolically active. The explanation why milk yield in dairy goats is relatively unaffected by CL (in contrast to dairy cows) remains to be established.

In conclusion, mammary development and remodelling prior to parturition and during lactation in goats are dependent on parity as well as late gestation management (dry period). Redevelopment of the mammary microvascular system is closely coordinated with that of the mammary epithelial cell population, and crucial for overall integrity of mammary gland function, which explains higher lactation persistency in PP compared to MP animals. Omitting the late gestation dry period in dairy goats did not depress milk production in the following lactation (in contrast to dairy cows) despite interference with peri-partum mammary (re)development, and mammary nutrient extraction was not improved. The reason for this species difference is interesting, but as yet unknown. Furthermore, the cellular events explaining the differential developmental patterns 1) in PP versus MP animals and 2) in mammary glands allowed a pre-partum dry period versus mammary glands continuously milked throughout late gestation still need to be resolved.

The decreasing mammary metabolic activity with progressing lactation appears to alter the sensitivity of the mammary gland towards variations in nutrient supply. Milk protein synthesis requires presence in the MEC of building blocks in the form of amino acids as well as energy in the form of ATP. An insufficient supply of amino acids to the mammary gland of dairy goats can be compensated in EL but perhaps not LL by increased mammary supply of energy, provided that this increased energy supply is in the form of nutrients like acetate, which is metabolized predominantly in pathways resulting in generation of ATP. The mammary gland may thus be relatively less sensitive towards variations in amino acid supply in EL compared to LL, but the underlying reason for this altered sensitivity during lactation remains to be established. This indicates that there could be scope for development of differential protein recommendations for ruminants in early and late lactation, and this issues should be pursued in future studies.

Sammendrag (Summary in Danish)

Mælkeproduktionen er generelt lav, men det er set at laktation persistensen er højere hos primiparous (PP) end hos multiparous (MP) drøvtyggere. Dette kan være relateret til forskelle i udvikling og opretholdelse af mælkekirtlernes funktion i yveret, dog er de underliggende mekanismer for dette er ikke klarlagt. Endvidere er mælkeproduktionen hos MP drøvtyggere ikke kun bestemt af dyrets genetiske ydeevne, men også af management strategier for varigheden af goldperioden og perioden omkring ny laktation. Goldperioden synes at være afgørende for MP lakterende drøvtyggere, idet antallet af mælkekirtlens epitelceller (MEC) effektivt kan udskiftes efterhånden som en laktation ophører, og være forudgående for opstarten af den næste laktation. Generelt set afhænger mælkeproduktion og ændringer i mælkeydelsen under laktationen af tre hovedfaktorer så som 1) antallet af MEC, som er påvirket af forholdet mellem celleproliferation og apoptose, 2) den sekretoriske aktivitet af disse celler, som er påvirket af deres differentiering, og 3) tilførsel af næringsstoffer og fjernelse af metaboliske affaldsprodukter via blodet. Formålet med dette PhD projekt var at undersøge følgende hypoteser at 1) forskelle mellem PP og MP dyr i forbindelse med mælkeproduktion og laktation persistens kan være relateret til forskelle i vækst af mælkekirtlen og dens remodellering under laktation, 2) faktorer, der er ansvarlige for intervenerer mælkekirtelens remodellering i en sammenhængende laktation, som inkluderer goldperioden til den efterfølgende laktation, er de samme faktorer, der også er involveret i bestemmelse af laktation persistens og forskelle i ydeevne mellem PP og MP dyr, 3) mælkeproteinsyntesen i den lakterende mælkekirtel vil være mindre sensitive overfor variationer af næringsstofforsyning i sen laktation sammenlignet med tidlig laktation, 4) et mindre underskud i proteinindholdet i diæten kan kompenseres ved tilførsel af energisubstrater (ATP) til at fastholde mælke(protein)syntesen.

Hypoteserne er adresseret i fire artikler baseret på tre forsøg udført i malkegeder. I forsøg 1 og 2, blev mælkekirtelens remodellering sammenlignet mellem PP og MP geder under laktation. I multiparous geder, var betydningen af goldperioden for mælkekirtelens remodellering yderligere bestemt ved en ensidig kirtel goldning ca. 9 uger før læmning, efterfulgt af en normal laktation (NL), samtidig med undladelse af goldperioden, hvorved den anden kirtel blev udsat for en forsat laktation (CL). I forsøg 3, blev betydningen af næringsstofforsyning for regulering af mælke(protein)syntese bestemt. Det blev gjort ved at tilføre ekstra næringsstoffer gennem intravaskulær iso-osmotic infusion af næringstoffer (essentielle

aminosyrer, acetat eller glukose) for at klarlægge effekten på mælkeydelse og optagelse af næringsstoffer i mælkekirtlen. Gederne blev fodret med en basal diæt, som henholdsvis dækkede ca. 90 % og 80 % af behovet for nettoenergi og absorberbare aminosyrer fra tyndtarmen.

Dette blev udført i tidlig (EL) og sen laktation (LL) for at bestemme ændringerne i mælkekirtlens sensitivitet overfor variationer i næringsstofforsyning med fremskreden laktation, og dermed kunne bestemme om et fysiologisk grundlag til udvikling af en særlig anbefaling af proteintildeling hos drøvtyggere. Mælkekirtelbiopsier blev udtaget i forsøg 1 og 2 fra begge mælkekirtler, og blodprøver blev udtaget i alle forsøg til bestemmelse af forskelle i arteriovenøse koncentration forskelle for hver mælkekirtel på forskellige stadier af drægtighed og laktation. Gentransskription i forbindelse med MEC turnover, lactogenesis, angiogenese og den vaskulære funktion blev målt via real time RT-PCR, karakterisering af mælkekirtelens morfologi (kvantitativ histologi), og celle turnover blev ydermere bestemt (TUNEL og Ki-67). Fuldblod blev analyseret for syre-base parametre, og plasma blev analyseret for indhold af væsentlige metabolitter og hormoner.

Forsøgsresultaterne viste, at indledningen af laktation i PP mælkekirtlen er forbundet med kirtlens udvikling og vækst i tidlig laktation, som fortsætter i en længere tidsperiode i forhold til MP mælkekirtler. Det er blevet vist, at gener regulerende for de dirigerede ændringer i mælkekirtelens epitelceller og den vaskulær funktion under laktation, stort set er det samme sæt gener, der forklarer forskellige udviklingsmønstre mellem PP og MP geder. Udviklingen af både vaskulær og mælkekirtlens epitelceller er afgørende for mælkeydelsen og kan forklare den højere rapporterede laktation persistens i PP geder i forhold til MP. Men den underliggende årsagsmekanisme til hvorfor udviklingen af mælkekirtlen hos PP dyr er en anden sammenlignet med MP, er endnu ikke blevet klarlagt. Der er på nuværende tidspunkt ingen specifik genom faktor(er), der kan forklare forskellene i mælkekirtlens udvikling og MEC overlevelse mellem NL og CL kirtler.

Vi har vist, at udeladelse af goldperioden er muligt, om end det indeholdte nogle komplikationer hos gederne, og var uden væsentlig indflydelse på mælkeydelsen i den efterfølgende laktation, som i modsætning til koen. Udskiftning af MEC blev undertrykt i CL mælkekirtler, hvilket resulterede i et mindre antal MEC i den efterfølgende laktation. På tidspunktet for læmning (og under hele laktationen), havde mælkekirtlerne under CL mindre alveoler, mere fuldt differentieret MEC og en væsentlig større kapillær andel sammenlignet

med mælkekirtler under NL. Herved lignede CL kirtlen en normalt lakterende kirtel i et mere avanceret stadium af laktation.

Ingen af de undersøgte genom faktorer kunne gøre rede for forskellene i mælkekirtlens udvikling og MEC overlevelse mellem NL og CL kirtler. Effektiviteten af mælkekirtlens udvinding af næringsstoffer var upåvirket af CL, og derfor vil flere MECs i en mere fuldt differentierede tilstand ikke nødvendigvis være mere metabolisk aktive. Forklaringen på hvorfor mælkeydelsen hos malkegeder er relativt upåvirket af CL (i modsætning til malkekøer) er endnu ikke klarlagt.

Det kan hermed konkluderes, at udviklingen og remodellering af mælkekirtlen før læmning og under laktation hos malkegeder afhænger af paritet samt management under sendrægtighed (goldperiode). Videreudvikling (udvikling) af mælkekirtelens mikrovaskulærsystem er nøje reguleret af antallet af mælkekirtlens epitelceller, og afgørende for overordnet integritet af mælkekirtlens funktion, hvilket forklarer den højere laktation persistens hos PP sammenlignet med MP dyr.

At undlade sendrægtighedens goldperiode hos malkegeder nedsætter ikke mælkeproduktionen i den efterfølgende laktation (i modsætning til malkekøer) på trods af interferens med mælkekirtel udvikling peri-partum, samt mælkekirtlens udvinding af næringsstoffer ikke var forbedret. Årsagen til denne artsforskel er interessant, men forsat ukendt. Det er forsat ønskeligt at identificere de cellulære forekomster, der kan forklare de forskellige udviklingsmæssige mønstre 1) hos PP versus MP dyr og 2) i mælkekirtlens tilladelse af en præ-læmning goldperiode versus mælkekirtlens forsat mælkeproduktion under den sene drægtighed. Mælkekirtlens nedsatte metaboliske aktivitet under progressiv laktation fremtræder, at kunne ændre mælkekirtlens sensitivitet overfor variationer i næringsstofforsyningen. Mælkeproteinsyntesen i MEC kræver tilstedeværelsen af byggesten i form af aminosyrer samt energi i form af ATP. En utilstrækkelig forsyning af aminosyrer til mælkekirtlen hos malkegeder kan kompenseres i EL, men måske ikke LL, ved øget energitilførsel til mælkekirtlen, forudsat at denne øgede energitilførsel er i form af næringsstoffer som acetat, der hovedsageligt omsættes til ATP. Mælkekirtlen må dermed være relativt mindre sensitive overfor variationer i aminosyretilførsel i EL forhold til LL, men den underliggende årsag til denne ændrede sensitivitet under laktation er endnu ikke klarlagt. Dette indikerer, at der kunne være mulighed for udvikling af forskellige proteinanbefalinger til drøvtyggere i tidlig og sen laktation, som er nærliggende at undersøge i fremtidige studier.

چکیده (Summary in Farsi)

در مجموع، نشخوارکنندگان در دوره شیردهی اوّل (نخستین زایمان) در مقایسه با شیردهی های بعدی (زایمان دوم به بعد)، میزان تولید شیر کمتر ولی با تداوم بیشتری دارند. این مسأله می تواند نشأت گرفته از تفاوتهای آنها در تکامل و نگهداری عملکرد غده پستانی باشد؛ هر چند، ساختارهای زیربنایی آن به درستی شناخته نشده اند. از این گذشته، تولید شیر در شیردهی دوم به بعد نه تنها با ظرفیت تکوینی دام، بلکه با عوامل مدیریتی مانند طول دوره خشکی ما بین دو دوره شیردهی متوالی سنجیده می شود. به نظر می رسد که دوره خشکی برای دوباره نوسازی مؤثر جمعیت سلولهای پوششی پستان در پایان یک دوره شیردهی و پیش از آغاز دوره شیردهی بعدی، ضروری است. در مجموع، میزان تولید شیر و تغییرات تراوش شیر در دوره شیردهی بستگی به سه عامل اصلی دارد: الف) تعداد سلولهای پوششی پستان، که منتج از موازنه مابین سرعت زایش و مرگ آن سلولهاست؛ ب) فعالیت ترشحی آن سلولها، که تحت تأثیر درجه تمایز آن سلولها می باشد؛ و ج) غذارسانی و خارج کردن مواد زائد ناشی از سوخت و ساز از طریق جریان خون. پایان نامه حاضر، بر آن دارد تا این فرضیه ها را مورد بررسی قرار دهد: الف) تفاوتهای دامهای در دوره اوّل و چندم (دوم به بعد) شیردهی در تولید شیر و تداوم شیردهی، ممکن است همچنین مرتبط با تفاوتها در رشد و تغییرات ساختاری پستان در طول دوره شیردهی باشد؛ ب) عوامل مسؤول تداخل با تغییرات ساختاری پستان در شیردوشی ممتد (در طول دوره خشکی تا دوره شیردهی بعدی)، همان عواملی هستند که درگیر در تعیین تفاوتهای عملکردی و تداومی شیردهی مابین دامهای در دوره اوّل و چندم (دوم به بعد) شیردهی می باشند؛ ج) ساخت پروتئین شیر در غده پستانی در انتهای دوره شیردهی در مقایسه با ابتدای دوره شیردهی، حساسیت کمتری در قبال تنوع منابع مغذی خواهد داشت؛ د) جهت ثبات میزان تولید (پروتئین) شیر، کمبودهای پروتئین به میزان کم در رژیم غذایی می تواند با تأمین مغذی های تولیدکننده انرژی (ATP) جبران شود.

این فرضیه ها در چهار مقاله بر اساس سه آزمایش بر روی بزهای شیری گزارش شده اند. در آزمایشهای اوّل و دوم، تغییرات پستانی در بزهای شیردهی اوّل و شیردهی دوم به بعد در طول دوره شیردهی مورد مقایسه قرار گرفته اند. در بزهای شیردهی دوم به بعد، اهمیت دوره خشکی جهت تغییرات پستانی مورد بررسی مضاعف قرار گرفته است؛ بطوریکه، با استفاده از خشک کردن یک طرفه یک غده تقریباً نه هفته پیش از زایمان، ادامه یافته با یک دوره شیردهی عادی؛ درحالی که، دوره شیردهی و به تبع آن شیردهی ممتد به غده دیگر تثبیت شده بود. در آزمایش سوم، اهمیت مواد غذایی در ساخت (پروتئین) شیر مورد بررسی قرار گرفته است. این [عمل] با تأمین مواد مغذی از طریق تزریق ممتد داخل وریدی محلولهای هم تراوا (ایزواسمز) (اسیدهای آمینه ، استات و گلوکز) و تعیین تأثیر آنها در میزان تولید شیر و برداشت (استفاده) پستان [از] مواد مغذی، انجام شده است. بزها با یک غذای پایه تأمین کننده تقریباً نود و هشتاد درصد مایحتاج خالص به ترتیب انرژی و اسیدآمینه قابل جذب از روده کوچک، مورد تغذیه قرار گرفتند. این [عمل] هم در اوایل و هم در اوآخر دوره شیردهی جهت بررسی تغییرات حساسیت پستان به تنوع مواد مغذی با پیشرفت [زمانی] دوره شیردهی انجام گرفته است ، و پیرو آن بررسی اینکه آیا پایه های فیزیولوژی جهت بسط توصیه های درجه بندی پروئین برای تغذیه نشخوارکنندگان، وجود دارد. بیوپسی های پستانی در آزمایش اوّل و دوم از هر دو غده پستانی، و نمونه های خونی در همه آزمایشها جهت تشخیص تفاوتهای غلظت سرخرگی-سیاهرگی در دو طرف هر غده پستانی در مراحل مختلف آبستنی-شیردهی انجام گرفته است. رونویسی ژنی مرتبط با تغییر و تبدیل سلولهای پوششی

پستان، شیرسازی، رگ سازی و فعالیتهای عروقی، به وسیله real time RT-PCR مورد ارزیابی قرار گرفته است. همچنین ریخت بافتی پستان (توسط بافت شناسی کمیتی) و تغییر و تبدیل سلولی (توسط ki-67 و TUNEL) مورد بررسی واقع شده است. همه خون مورد آنالیز پارامترهای اسیدی-بازی، و پلاسمای آن مورد آنالیز مقادیر متابولیتها و هورمونهای کلیدی قرار گرفته اند.

نتایج نشان می دهد که آغاز شیردهی در پستانهای دوره شیردهی اوّل با تکامل و رشد غده در اوایل دوره شیردهی مرتبط است، که در مقایسه با پستانهای شیردهی دوم به بعد، به مدت طولانی تری ادامه می یابد. شواهدی ارائه شده است که مجموعه ژنهای تنظیم کننده این تغییرات موزون در سلولهای پوششی و فعالیت عروقی پستان در طول دوره شیردهی، به طور اساسی همان مجموعه ژنهایی هستند که تفاوتهای ساختارهای تکاملی بین دوره شیردهی اوّل و دوم به بعد را تشریح می کنند. بنابراین، هم عروق و هم سلولهای پوششی پستان، هر دو مشخص کننده کارآیی شیردهی و همینطور بالاتر بودن تداوم شیردهی در بزهای شیردهی اوّل در مقایسه با بزهای شیردهی دوم به بعد هستند. امّا علت اصلی که چرا تکامل پستان در شیردهی اوّل مسیر متفاوتی در مقایسه با شیردهی دوم به بعد دارد، نیاز به تحقیق بیشتری دارد. هیچ عامل ژنی خاصی که بتواند جهت تفاوتهای تکاملی پستان و ماندگاری سلولهای پوششی پستان مابین دوره شیردهی اوّل و دوم به بعد به حساب آید، پیدا نشد.

ما ثابت کردیم که حذف دوره خشکی هرچند با مقداری مشکل، بدون تغییر عمده ای بر تولید شیر در بز، در مقایسه با گاو، امکان پذیر است. نوسازی سلولهای پوششی پستان در پستانهای بطور ممتد شیردوشی شده، سرکوب گشته، که باعث ایجاد جمعیت کوچکتری از سلولهای پوششی پستان در دوره شیردهی بعدی شده است. در زمان زایمان (و در طول شیردهی)، غده پستانی بطور ممتد شیردوشی شده، آلوئولهای کوچتر، سلولهای پوششی بطور کامل تمایزیافته بیشتر و همینطور بخش بزرگتری از مویرگها در مقایسه با غده های شیردوشی شده بطور عادی، داشتند؛ و بنابراین، غده بطور ممتد شیردوشی شده نمایانگر یک غده بطور عادی شیردوشی شده ای است که در مرحله پیشرفته تری از شیردهی قرار دارد. هیچکدام از عوامل ژنی مورد مطالعه قرارگرفته، نمایانگر تفاوتهای تکاملی پستان و ماندگاری سلولهای پوششی پستان مابین غده بطور ممتد شیردوشی و غده بطور عادی شیردوشی شده نبودند. راندمان پستان در استخراج مواد مغذی تحت تأثیر شیردوشی ممتد قرار نگرفت، و بنابراین، تعداد بیشتر سلولهای بطورکامل تمایزیافته پوششی پستان، لزوماً فعالیت سوخت و سازی بالاتری ندارند. توضیح اینکه چرا تولید شیر در بزهای شیری تحت شیردوشی ممتد (در مقایسه با گاوهای شیری) تحت تأثیر قرار نگرفته اند، نیاز به تحقیق بیشتری دارد.

به عنوان نتیجه گیری، تکامل و تغییر ساختار پستان قبل از زایمان و در طول دوره شیردهی در بزها، مستقل از زایمان [چندم] (دوره شیردهی چندم) و همینطور مدیریت اوآخر دوره آبستنی (دوره خشکی) می باشد. تکامل دوباره سیستم عروقی پستان بطور نزدیکی هماهنگ است با جمعیت سلولهای پوششی پستان و عملکرد بی عیب غده پستانی، که توضیحی است برای تداوم شیردهی بیشتر در دوره شیردهی اوّل در مقایسه با دوره شیردهی دوم به بعد. حذف دوره خشکی اوآخر آبستنی در بزهای شیری (در مقایسه با گاوهای شیری)، تولید شیر در دوره شیردهی بعدی را علی رغم تداخل با تکامل دوباره پستان در دوره پیش از زایمان، کم نکرد. علت تفاوت این دو گونه جالب توجه، امّا ناشناخته است. به علاوه، اتفاقات سلولی توضیح دهنده تفاوتهای الگوهای تکاملی در الف) دامهای شیردهی اوّل و دوم به بعد، و ب) در غدد پستانی که دوره خشکی پیش از زایمان داشتند دربرابر غدد پستانی که بطور ممتد در طول آبستنی شیردوشی شده اند، هنوز احتیاج به حل شدن دارد.

به نظر می رسد که کاهش فعالیتهای سوخت و سازی پستان با پیشرفت [زمانی] شیردهی، حساسیتهای غده پستان به تنوع مواد مغذی را تغییر می دهد. ساخت پروتئین شیر احتیاج به حضور بلوکهای ساختمانی به صورت اسید آمینه و همینطور انرژی از نوع ATP در سلولهای پوششی پستان دارد. تأمین ناکافی اسیدهای آمینه به غده پستان بزهای شیری، می تواند با افزایش تأمین انرژی به پستان در اوایل دوره شیردهی و نه در اوآخر دوره شیردهی، جبران شود؛ با این تضمین که این افزایش تأمین انرژی به شکل مغذی هایی مثل استات باشد، که به صورت عمده در مسیرهایی که منجر به تولید ATP است، سوخته می شوند. بنابراین، غده پستانی ممکن است به تنوع منابع تأمین اسید آمینه در اوایل دوره شیردهی در مقایسه با اوآخر دوره شیردهی، حساسیت کمتری داشته باشد؛ امّا، علت این تغییر حساسیت در طول دوره شیردهی نیاز به تحقیق [بیشتر] دارد. این بیانگر آن [واقیت] است که بسط توصیه های پروتئینی برای نشخوارکنندگان در اوایل و اوآخر دوره شیردهی جای کار بسیار دارد، و این مقوله می بایست در مطالعات آینده مورد پیگیری قرار گیرد.

Introduction

The level of milk production and the changes in milk yield over the course of lactation, depend on: A) the number of milk synthesizing mammary epithelial cells (**MEC**), which is affected by the balance between the rate of epithelial cell proliferation and apoptosis (Capuco et al., 2003), B) the secretory activity of these cells, which is affected by their degree of differentiation (Akers et al., 2006), and finally C) the provision of nutrients and removal of waste products via the vascular system essential for sustaining milk synthesis. These determining factors are, in general, affected by major factors like the genetic capacity of the animal, the parity number, management factors such as duration of the dry period in between successive lactations, and the amount and kind of nutrients supplied to the mammary gland.

Mammary growth up until the first lactation has been studied quite extensively, whilst remodeling of the mammary gland during lactation and particularly in between successive lactations has received far less attention. Much remains to be known about how and by which mechanisms remodelling of the MEC and particularly the mammary microvasculature occurs in late gestation, and how it relates to lactational performance, MEC secretory activity and microvascular function in the subsequent lactation. The efficiency of mammary (re)modelling/development taking place pre-partum impact lactation performance post-partum and also lactation persistency. Understanding how this regulation takes place and identifying the key regulatory factors might therefore help to put such knowledge into use in future attempts to improve lactational performance and persistency in dairy animals.

Generally, multiparous (**MP**) ruminants have a higher milk yield but lower lactation persistency compared to primiparous (**PP**) ones in their first lactation (Miller et al., 2006). This may be related to differences in development and maintenance of mammary gland function, and it has been reported that mammary growth continues longer post-partum into lactation in PP compared to MP goats (Anderson et al., 1981; Knight and Peaker, 1984) and cows (Miller et al., 2006). However, the underlying regulatory mechanisms for these differences in developmental patterns are not well understood. Metabolic status through the lactation period in an animal during its first lactation is different from that in subsequent lactation as the animal matures, since nutrients in PP animals are prioritized not only for lactation but also for the continued growth of the animal (Wathes et al., 2007). Therefore, comparing PP vs MP animals could improve our insight into the regulatory mechanisms that

determine mammary remodelling in late gestation and during lactation, and hence potentially identify important factors determining lactation performance and persistency.

In MP dairy cows, renewal of the MEC population during the dry period in between successive lactations appears to be crucial to ensure optimal milk production in the following lactation (Capuco et al., 1997; Madsen et al., 2008; Remond et al., 1997; Swanson, 1965). Omitting the dry period and milking dairy cows continuously from one lactation into the next will interfere with MEC cell renewal (Annen et al., 2008; Capuco et al., 1997; Sorensen et al., 2006) and has been reported to result in a substantial depression (up to 40%) of milk production in the next lactation. It remains to be established how and by which mechanisms continuous milking during late gestation can interfere with the MEC renewal, preventing the MEC population from fully expressing their production potential. The continuously lactating mammary gland does appear to be able to respond to systemic lactogenic signals initiating a new lactation with increased milk production around parturition (Caja et al., 2006; Madsen et al., 2008), and the MEC in the CL gland are capable of undergoing cell renewal (Annen et al., 2007), and it remains to be established why a simple thing like continued removal of milk is interfering with restoration of mammary synthetic capacity in late gestation, when the processes involved in this restoration are apparently not inhibited.

Despite the importance of the mammary microvascular system for sustenance of milk synthesis by providing the nutrients and oxygen to MEC and removing the metabolic waste products, remodelling of this mammary tissue compartment during the late gestation and following lactation period has received virtually no attention in normal mammary development of dairy animals (Akers, 2002; Djonov et al., 2001). An experimental animal model, with unilateral continuous milking (continuous lactation, **CL**) of one udder half in the late gestation period whilst drying off the other udder half (normal lactation, **NL**), would be useful to get improved insight into the local key regulatory factors involved in the remodelling of the mammary gland in the late gestation period and the relative importance of the MEC and microvascular compartments in determining lactation performance in the following lactation.

Not only mammary remodelling (capacity of the mammary gland) is important for lactation performance, but also the extent to which this capacity can be exploited. Provision of nutrients to the mammary gland is an important factor in this respect. Synthetic capacity of the mammary gland and hence milk production change from early towards late lactation. There are indications suggesting that this is associated with an altered sensitivity of the

mammary gland towards variations in e.g. amino acid supply, since correlations between arterial concentrations and arterio-venous differences (efficiency of mammary uptake) for individual amino acids differed in early compared to late lactation in dairy goats fed different levels of lysine and methionine (Madsen et al., 2005).

Protein synthesis in the mammary gland as in any other tissues relies on energy in the form of ATP, and both amino acid and energy supply to the mammary gland could thus limit milk protein synthesis. This may in part explain why increased supply of a number of specific amino acids have been found to have a positive effect on milk protein production in some situations (Hanigan et al., 2001; Rulquin et al., 1993; Weekes et al., 2006), but not always (Seymour et al., 1990), when supply of other energy yielding substrates were not increased. Acetate is utilized in oxidative phosphorylation of adenosine nucleosides, which results in the generation of ATP (Forsberg et al., 1984; Scott et al., 1976), whereas glucose is oxidized mainly through the pentose phosphate pathway to yield NADPH required for *de novo* fatty acid synthesis (lipogenesis) (Chaiyabutr et al., 1980; Chaiyabutr et al., 2008). If mammary energy supply is a main determinant for protein synthesis, it seems theoretically possible to be able to substitute amino acids to some extent in the diet with an energy yielding substrate to provide extra ATP in the mammary gland and improve utilisation of the amino acids available, but the extent to which this is possible may depend on the stage of lactation and the sensitivity of the mammary gland towards nutrient supply.

Altogether it can be hypothesized that:

1) differences between PP and MP animals with respect to milk production and lactation persistency may be related to differences in mammary growth and remodelling also during lactation,

2) the factors responsible for interfering with mammary remodelling in CL are the same factors involved also in determination of lactation persistency and performance differences between PP and MP animals,

3) milk protein synthesis in the lactating mammary gland will be less sensitive towards variations in nutrient supply in late compared to early lactation,

4) minor deficiencies in dietary provision of protein can be compensated by provision of energy (ATP) yielding substrates to sustain milk (protein) synthesis.

The aims of the experimental work in dairy goats were to:

I) determine if parity number (PP vs MP) and dry period (CL vs NL) impact mammary gland synthetic capacity exclusively through interference with turn over and function of the MEC, or if mammary remodelling of the micro-vascular system plays an equally important role,
II) identify the essential regulatory mechanisms (factors) explaining the differences among those different models,
III) if milk synthesis is more sensitive towards changes in nutrient provision in early lactation compared with late lactation, and
IV) elucidate whether provision of energy (ATP) yielding substrates can compensate for an insufficient AA supply to the mammary gland and hence improve overall utilization of AA for milk protein synthesis, and if acetate is more efficient than glucose in that respect.

Altogether, such comparative studies can provide evidence needed to find new avenues for the genetic selection and/or management of the dairy animals to increase the economical profitability in dairy production industry. In recent years, consumers' increased demand for protein has promoted the value of milk protein production, and environmental concerns have placed increased focus on nitrogen utilization in farm animals. There might be a prospect for differentiating protein recommendations for ruminants across the lactation period, if milk protein production can indeed be sustained in certain stages of lactation through optimisation of the provision of energy yielding substrates relative to amino acids.

Background

In order to improve the lactational performance of dairy animals in the future, it is important to know what factors limit the production potential of the animal during lactation. It is important in this respect to understand the mechanisms that impact on mammary gland synthetic capacity through interference with the turnover and function of the MEC and the mammary microvasculature during the gestation-lactation period. This section begins with a brief outline of the mammary gland and its development, focussing on epithelial cell turnover and micro-vascularisation, and on the remodelling of the mammary gland that takes place over the course of gestation-lactation. An effort is made to identify the crucial factors determining synthetic capacity and hence production potential and lactation persistency. In addition, the importance of the nutrient supply for milk synthesis and hence the utilisation of the synthetic capacity of the mammary gland, along with the sensitivity of the mammary gland towards changes in nutrient provision at different time points in lactation, will be discussed.

The mammary gland

The mammary gland is a secondary sexual organ and a part of the reproductive system (Sejrsen, 1994). It has the same overall structure in all ruminant species, with minor differences (goats and sheep have two mammary glands in the udder, as opposed to four in cows. The two udder halves are independent of each other).

The mammary gland is composed of lobulo-alveolar clusters, emerging from a branching network of ducts which are made by polarized single-layer epithelial cells (McManaman and Neville, 2003). Myoepithelial cells and a vascularized connective-tissue stroma containing adipocytes and fibroblasts cover the alveoli in the mammary gland. Figure 1. gives a schematic illustration of the mammary alveolar system.

MEC are the milk synthesizing units in the mammary gland, determining the quality and quantity of milk secretion (Akers, 2000). Milk yield is a function of the number of these cells as well as their activity. Their number depends on the stage at which the mammary gland has developed to, and hence, the previous rate of cell turnover in the gland. At the same time, the MEC's productivity depends on their differentiation as well as their blood supply and on the efficiency of exchange of nutrients and waste products across the capillary-MEC barrier. In the section that follows the diagram, the key issues pertaining to the mammary gland - such as

its development, cell turnover, micro-vascularization, blood supply and metabolism - are briefly described.

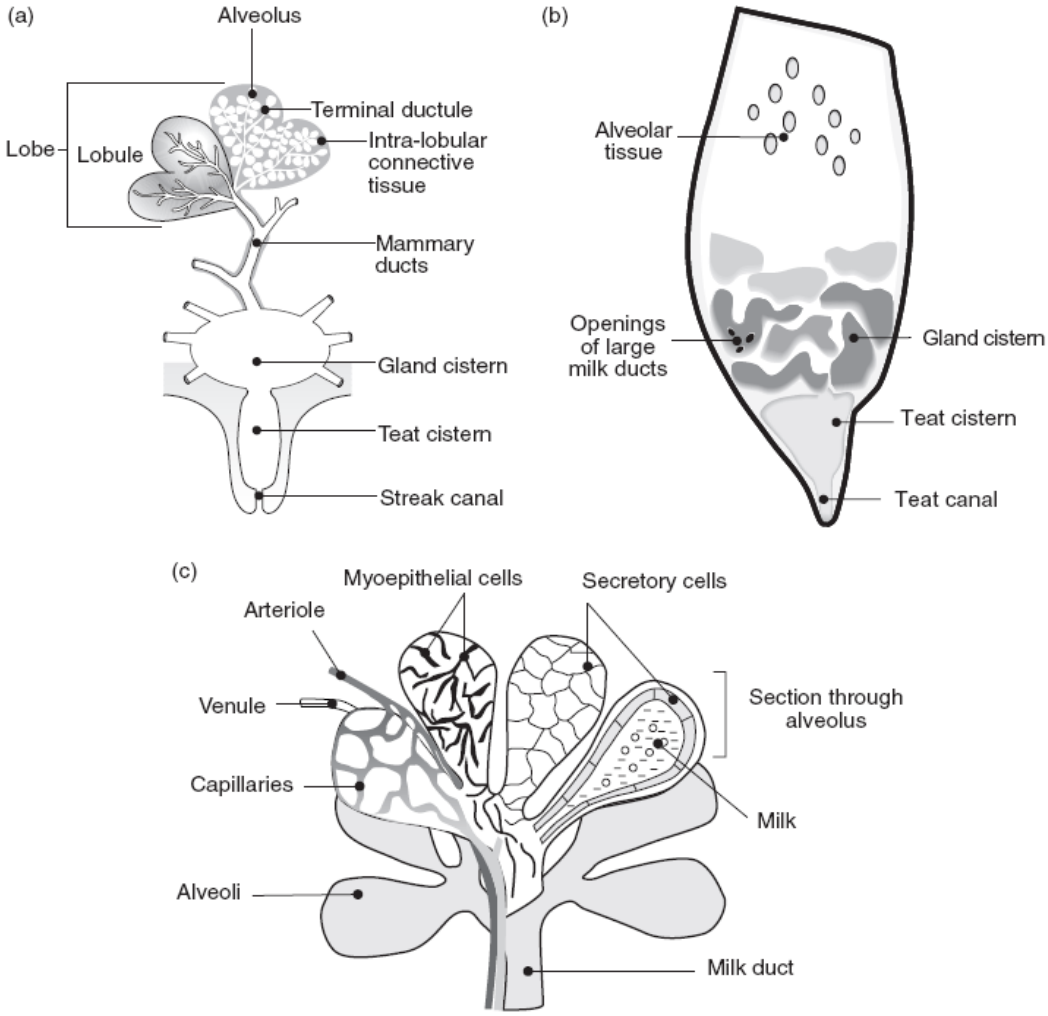


Figure 1- Schematic diagram of mammary duct and lobular-alveolar system: a) overall: b) cross-section; c) surface and internal anatomy of alveoli (Lawrence and Fowler, 2002).

Development

The development of the mammary gland may be described following its different stages, starting in fetal life, continuing over puberty, and subsequently during the gestation and lactation periods. In bovine biology, the growth of the mammary gland starts being isometric with other body organs and becomes allometric before puberty, with the first few oestrus cycles (Sejrsen, 1994; Sheffield, 1988). After puberty, its growth again becomes isometric, growing at a rate parallel to the rest of the body (Sinha and Tucker, 1969). Pubertal mammary

growth is important for the future development and ultimate milk-producing capacity of the mammary gland (Radcliff et al., 2000; Sejrsen, 1994), and any changes in this period are liable to affect the mammary gland potential for milk synthesis in the following lactation periods.

In dairy heifers, parenchymal growth doubles from day 260 of pregnancy to day 49 of lactation; however, the amount of DNA does not change (Keys et al., 1989). Norgaard et al. (2008a) have reported that in sheep the major phase of mammary redevelopment (growth and differentiation between successive lactations) takes place during late pregnancy, within the last 38 days pre-partum, during which period parenchyma in mammary tissue have an eightfold increase in weight and an MEC increase from 5 to 19 %. In general, the major element of mammary growth in ruminants (mammogenesis) occurs in late pregnancy and before the onset of lactation (pre-partum period, lactogenesis): it is then that the formation of its lobulo-alveolar structure is initiated (Akers, 2002; Erb, 1977; Tucker, 1987). It has been reported that in primiparous dairy goats (Anderson et al., 1981; Knight and Peaker, 1984) and cows (Miller et al., 2006), mammary growth also continues into the first early lactation, although with a lower speed than during gestation. Mainly, this is a growth in parenchyma, coinciding with a decreased proportion of stroma (Akers et al., 2006; ELsayed et al., 2009). Compared to the mammary development in primiparous ruminants, mammary re-development in second gestation animals has been shown to have a faster growth of parenchymal volume, especially in late gestation when the gland dries out and prepares for the new lactation (Knight and Wilde, 1993). Within the mammary parenchyma, the differentiation of the MEC starts a few days pre-partum and continues till the subsequent peak lactation (Annen et al., 2007). When reaching parturition, this differentiation seems to be more pronounced in multiparous than in primiparous ruminants (Miller et al., 2006), so that less poorly differentiated MEC are evident in the mammary gland of multiparous cows at parturition compared to their primiparous counterparts (Ellis and Capuco, 2002).

Metabolic status during the lactation period in the first lactating mammary gland differs from that in the more mature ones, as the nutrients in primiparous (**PP**) animals are prioritized not only for lactation but also for the continued growth of the animal (Wathes et al., 2007). Furthermore, multiparous (**MP**) ruminants have a higher milk yield but lower lactation persistency compared to PP ones (Miller et al., 2006). This may be related to differences in the development and maintenance of the mammary gland function; as yet the underlying mechanisms are not well understood. The differential mammary remodelling in PP compared

to MP animals may be related not only to the MEC population but also to the vascular system required for nutrient supply and the removal of metabolic waste products. Angiogenesis - the formation of new capillaries from pre-existing blood vessels - is a biological process known in other species to be associated with mammary remodelling (Matsumoto et al., 1992). Its function is to accommodate highly variable requirements for nutrients and oxygenation with respect to changes in mammary tissue composition and metabolic activity (Djonov et al., 2001). The process has received virtually no attention in discussions of the normal mammary development of dairy animals (Akers, 2002), although the coordinated development of mammary vascular and epithelial cell function must be assumed to be of the utmost importance for normal mammary gland function and performance. Vascular function and remodelling may not be the same in PP and MP glands since the pre-partum development in either case could be different, since PP animals have not had a previous lactation with a fully developed mammary gland. So it is important to elucidate how and to what extent the differences in lactational performance between PP and MP mammary glands are related to the time-course of development and maintenance not only of the MEC population but also of the mammary vasculature which sustains synthetic activity. In addition, we need to identify the underlying regulatory factors or mechanisms responsible for such developmental differences between PP and MP mammary glands as exist. Knowledge about the mechanisms responsible for the regulation of MEC renewal as well as the remodelling of the mammary microvasculature can be of assistance in finding new ways to improve lactation persistency and overall lactational performance, to the ultimate economic benefit of producers.

In multiparous dairy herds, it has been shown that the mammary glands of cows continuously milked throughout the pre-parturient period have fewer secretory cells prior to calving, and a more stable epithelia-to-lumen ratio, than do cows that were dried off in that period, even though the total parenchymal mass was not affected by the pre-partum milking (Capuco et al., 1997). In addition, differentiation of MEC seems to be more important than their number in early lactation; this is what causes the differences in productivity between dairy and beef animals (Akers et al., 2006). According to Wilde et al. (1986), the increase in milk yield observed after parturition in early lactation can be explained by a higher rate of cell differentiation resulting in a higher cell number and synthetic capacity in the gland. Likewise, it is suggested that the reduction in milk yield after peak of lactation can be accounted for by a decrease in MEC number rather than because of secretory activity per cell. So, depending on

the stage of lactation, MEC number and/or its secretory ability seem to be the main determinants of mammary gland synthetic capacity.

In multiparous ruminants, the dry period is necessary for a full restoration of mammary synthetic capacity in the next lactation (Capuco et al., 1997; Remond and Bonnefoy, 1997; Swanson, 1965). According to previous studies (Akers, 2002; Bachman and Schairer, 2003; Capuco et al., 1997; Hurley, 1989; Smith and Todhunter, 1985), the dry period can be divided into three stages: 1) early dry period, when milk synthesis comes to an end and the gland status changes from lactating to non-lactating in the absence of the suckling/milking stimulus. At this stage, involution is dominant with regression of the MEC and gland structures, involving apoptosis and tissue remodelling; 2) mid dry period, when the involution process is complete and the non-lactating gland morphology remains relatively stable, and, 3) late dry period, when redevelopment of the mammary tissue and lactogenesis happen prior to and around parturition and initiation of the next lactation. The dry period appears to be essential for dairy animals to ensure that the MEC population can be effectively renewed at the end of one lactation and prior to onset of the next (Capuco et al., 1997); it is crucial in ensuring optimal milk production in the following lactation in dairy cows (Madsen et al., 2008; Remond et al., 1997; Swanson, 1965). It has been shown that omitting the dry period while milking dairy cows continuously from one lactation into the next will interfere with MEC cell renewal (Annen et al., 2008; Capuco et al., 1997; Sorensen et al., 2006) and result in milk yield depressions of 20% or more in the following lactation (Madsen et al., 2008; Remond et al., 1997; Swanson, 1965). Relatively few studies have been performed on continuous lactation (**CL**) in dairy goats, and the impact of omitting the dry period on milk production in the next lactation in this species appears to be less clear. Fowler et al. (1991) and Mackenzie (1967) report that CL has no negative effect on milk yield in subsequent lactations. However, the experimental design by Fowler et al (1991) included an unusually long dry period of 24 weeks. In contrast to this, Caja et al. (2006) reported decreases in milk production of 29% in dairy goats in response to omitting the dry period. To our knowledge, there have been no previous studies showing how CL impacts on MEC turnover in the goat, and the impact of CL on the remodelling of the microvascular system has not been studied in any species so far.

Cell turnover

Rather than the size of mammary gland, the best indicator of its synthetic capacity is the relative proportion of its secretory tissue, ie. MEC (Boutinaud et al., 2004; Capuco et al.,

2003; Knight, 2000). The MEC population depends on an earlier balance between cell proliferation and apoptosis, and different degrees of activity in these two events involved in cell turnover could issue in an equal number of cells (Capuco et al., 2001). Knight and Peaker (1984) have reported that each stage of gestation-lactation in dairy goats has its own characteristics with respect to changes in secretory cell number and productivity (fig. 2). As discussed above, the dry period appears to be essential for dairy animals to ensure that the MEC population can be effectively renewed as one lactation comes to the end and another one begins (Capuco et al., 1997). Pre-partum milking does not prevent cell turnover (renewal) in the late gestation period, but obviously limits the extent of such a renewal (Capuco et al., 1997). The MEC is also capable of responding to lactogenic signals around the time of parturition; it may initiate a new lactation, since parturition is associated with an increase in milk production resembling the pattern of change in milk production following a normal dry period (Madsen et al., 2008; Remond et al., 1997). It has as yet not been possible to establish through which key regulatory factors or processes CL interferes with mammary redevelopment, and why continued removal of milk throughout the late gestation period is incompatible with the re-establishment of full lactational capacity in the subsequent lactation (Caja et al., 2006; Madsen et al., 2008). Yet these underlying mechanisms and regulatory factors are obviously crucial for the remodelling of the mammary gland during late gestation in order to allow it to reach the optimum lactational performance.

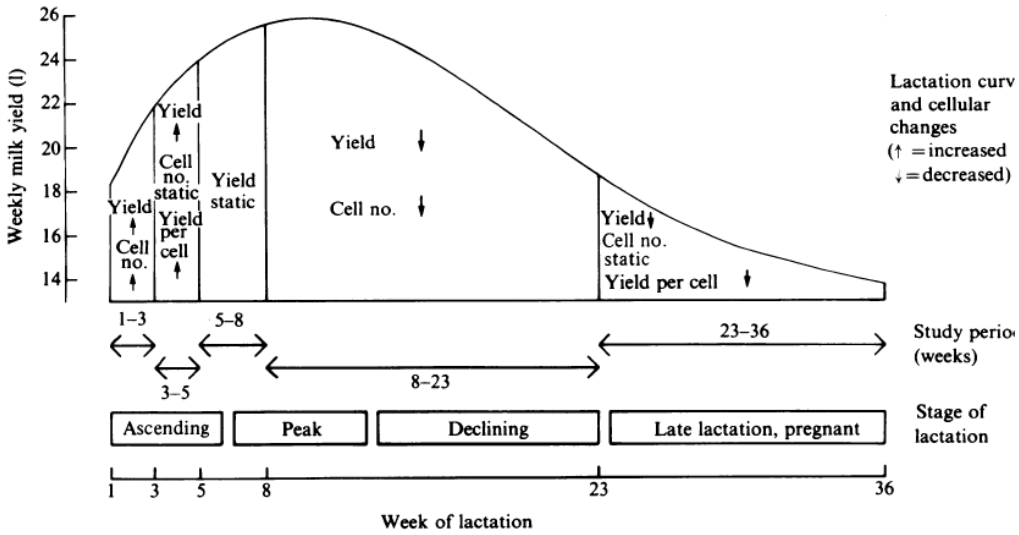


Figure 2- Diagram of lactation curve, mammary secretory cell number and milk yield per cell during lactation in dairy goats (Knight and Peaker, 1984).

In the following section the general concepts of cell proliferation and apoptosis are explained briefly, and key regulatory factors are identified that could potentially be affected by CL as well as being involved in the different developmental patterns observed in the mammary glands of PP *vs* MP animals.

Proliferation concepts. Proliferation is a process by which a new cell is produced from a pre-existing cell. The cell cycle of mitotic cells has two major phases (King et al., 1996; Vermeulen et al., 2003): 1) M (mitosis), the process of nuclear division, including prophase, metaphase, anaphase and telophase; and, 2) interphase, the process between two M phases, including G_0 (resting), G_1 (first gap), S (synthesis) and G_2 (second gap).
New cells resulting from mitosis either go directly to G_1 or else enter G_0. In G_1, the cells grow in size, become resting or get prepared for DNA synthesis. After this they move into the G_0 phase and rest until the time when they are able to get back into the cycle, either returning back to the G_1 phase again, or else continuing into the S phase where the DNA is duplicated. After the S phase, the cell enters G_2 where it synthesises the needed proteins to progress to M phase. Control mechanisms for these phase transitions play an important role in cell proliferation (Norgaard, 2007). The mechanisms in question require a variety of proteins to be able to regulate the progress of cell cycle towards proliferation.

Apoptosis. Apoptosis is a programmed process leading to the death of a cell. Its mechanisms work to limit cell numbers, excluding unnecessary and aged cells, and eliminating potentially detrimental classes of cell (Strange et al., 1992). Some characteristics of apoptotic cells can be distinguished microscopically such as cellular shrinkage, cellular budding, condensed nuclei/chromatin/cytoplasm, fragmented nuclei, and unsettled plasma membrane including apoptotic bodies (cell organelles and/or nuclear material surrounded by an intact plasma membrane) which have been phagocytised by adjacent cells and macrophages (Van Cruchten and Van den Broeck, 2002). Furthermore, what makes apoptosis different from other cell death processes such as necrosis is that, in apoptosis, no inflammatory responses are visible, nor is membrane integrity lost (Proskuryakov et al., 2003; Van Cruchten and Van den Broeck, 2002). Apoptosis uses two regulatory pathways (Norgaard, 2007): 1) extrinsic, by which the binding of extra-cellular proteins to death receptors on the cell surface activates the process (Maher et al., 2002); and, 2) intrinsic, where mitochondia plays a crucial function in cell survival and cytochrome c release (Waterhouse et al., 2002). In general, the initiation of

apoptosis involves particular stages of cell differentiation and thereby a limitation in the factors required for cell viability (Strange et al., 1992; Williams et al., 1990).

In the following section, factors believed to be important determinants in cell turnover and differentiation in the MEC, are introduced. These could potentially be involved in determining differences in remodelling the mammary gland 1) subjected to CL or else allowed a dry period (NL) in late gestation and 2) between PP and MP animals.

BCL2 and BAX. It seems clear that BCL2 and BAX have independent functions as survival and death factors, respectively (Reed, 1998). The expression of the pro-apoptotic agent BAX and the anti-apoptotic agent BCL2 has been studied in the mammary gland of dairy cows (Norgaard et al., 2008b) and of goats (Wareski et al., 2001). In dairy goats, Wareski et al. (2001) reported the lowest expression of BAX during peak lactation and the correspondingly highest expression in late lactation and the dry period; at the same time, there was a gradual increase of BCL2 expression from early to late lactation and evidence of it remained high during the dry period. The BAX/BCL2 ratio in the mitochondrial membrane seems to determine whether a cell undergoes apoptosis or survives (Schultz and Harrington, 2003). It follows from this that their expressional changes might help account for the level of cell turnover in the mammary gland.

Cyclin D1. Cyclin D1 (CCND1) has a role in the regulation/initiation of cell proliferation, and its expression is essential for cell cycle progression past the restriction point in G1 into the S phase. Its continuous synthesis is dependent on persistent growth factor stimulation (Norgaard et al., 2008a; Sherr, 1995). CCND1 has been studied in the mammary glands of dairy cows (Norgaard et al., 2008b) and of sheep (Norgaard et al., 2008a), where the results show a higher expression during gestation - specifically during the dry period - compared to lactation.

IGF family. IGF1 plays an important role as an anti-apoptotic survival factor in cell turnover during mammary gland development and remodelling during involution (Allan et al., 2004). Its receptor impacts growth and inhibits apoptosis (Baumrucker and Erondu, 2000). In general, the interaction of IGF1 and its receptor promotes cell proliferation and survival (Sell et al., 1995). Sell et al. (1995) have demonstrated the role of IGF1R as an inhibitor of apoptosis, independent of its mitogenic actions. IGF binding protein 3 (IGFBP3), a multi-functional protein, acts as a pro-apoptotic factor either independently from IGF1, or else serving as a transporter of IGF1, which in the mammary gland can be released from IGFBP3

in order to exert its action after binding of the IGF1-IGFBP3 complex to lactoferrin (Baumrucker et al., 2003; Butt and Williams, 2001; Grill and Cohick, 2000). Since it possesses the ability to inhibit cell proliferation and IGF1 action, it is thought that IGFBP5 may induce apoptosis in the mammary gland (Allan et al., 2004). In general, the IGF1 family members could play important roles in mammary remodelling, as their presence and actions are involved in related processes.

TGFB family. TGFB1 is another anti-proliferative and apoptogenic factor for MEC (Wareski et al., 2001); its two receptors, 1 and 2, mediate its effects (Akers, 2006). It seems that TGFB1 expression in goat mammary gland is low during early lactation, increases in late lactation and remains high during the dry period (Wareski et al., 2001); whereas in heifers its expression is low during lactation and high during involution (Plath et al., 1997). It seems to follow from this that it might play an important role in the dry period when the main part of mammary involution occurs.

Prolactin receptor. The prolactin receptor (PRLR) activates several pathways associated with proliferation, differentiation, and lactogenesis after binding with prolactin (Wall et al., 2006). While it has been suggested that the concentration of prolactin in plasma may be of less importance for development of mammary gland in cows, the level of its receptor in the mammary gland could well be of importance in mammary cell turnover and differentiation in ewes (Cassy et al., 2000) and in rodents (Neville et al., 2002).

Alpha-lactalbumin. Alpha-lactalbumin (LALBA), a key enzyme in lactose synthesis, is a good indicator of lactogenesis as its expression increases sharply around the time of parturition when lactation is initiated (Forsyth and Neville, 2009; McFadden et al., 1987). The enzyme is also known to be a pro-apoptotic protein (Riley et al., 2008).

Lactoferrin. Lactoferrin (LTF) is known to be involved in the immune system as well as helping govern the induction of cellular growth and differentiation (Ward et al., 2005). It is highly expressed during late pregnancy, involution and infections (Baumrucker, 2005). Further, it has been shown to be a specific receptor for IGFBP3, while binding of the IGF1-BP3 complex to lactoferrin will reduce the affinity of BP3 for IGF1(Baumrucker et al., 2003). In this way lactoferrin also plays a role in directing the action of systemic IGF1 towards the mammary gland.

Leptin. The inhibitory role of leptin on proliferation of mammary epithelial cells has been suggested by Silva et al. (2002). Leptin levels in milk have been reported to increase markedly two days after parturition in dairy goats and independently of changes in plasma

leptin (Chilliard et al., 2001; Rasmussen et al., 2008); this led to the suggestion that leptin and its receptor may play a role in mammary development and function in the early postpartum period in goats.

Microvascularization

Angiogenesis is a process whereby new capillaries are formed from pre-existing blood vessels (Djonov et al., 2001). As explained above, the process is important not only for mammary growth, but also for the maintenance and function of the mammary gland (Pepper et al., 2000a). According to an unproved hypothesis put forward by Akers (2002) concerning ruminants, and as also indicated in studies about rodents (Nishinak, 1970; Soemarwoto and Bern, 1958), steroid hormones such as estrogen and progestrone may stimulate growth of the mammary microvasculature as well as the mammary parenchyma. In most organs the angiogenic potential is only triggered in response to injury, whereas in the mammary parenchyma, the microvasculature is persistently regulated (Djonov et al., 2001). Mammary microvasculature indirectly plays a very important role for milk synthesis by providing nutrients and oxygen to the MEC essential to sustain milk synthesis, and by removing metabolic waste products (Djonov et al., 2001). Based on studies in mice (Djonov et al., 2001; Matsumoto et al., 1992), it seems that regression of mammary microvasculature mainly occurs during involution in late gestation pre-partum, while angiogenesis, and the meandering of the capillaries surrounding the alveoli, occurs mainly during lactation. It has been suggested that this meandering of capillaries is advantageous in increasing the microvasculature contact with the alveoli, which is most extensive at the peak of lactation in mice (Matsumoto et al., 1992). Pepper et al. (2000a) proposed the MEC as the main source of angiogenic factors in the mammary gland, which ensures a coordinated development of the microvasculature according to development of the MEC population. Djonov et al. (2001) have suggested that prepartum regression of the endothelium in the mouse mammary gland could be related to, or even a consequence of, MEC involution. Whatever comes first, either vascular or alveolar degeneration, it seems evident that the function and integrity of the MEC is dependent on and closely coordinated with the vascular system responsible for the provision of nutrients and the removal of waste products, all of which are essential to sustain MEC metabolism. Yet despite the importance of mammary microvasculature surrounding alveoli, there is virtually no information available for dairy animals regarding the normal remodelling of the mammary microvasculature during the gestation-lactation period (Akers,

2002; Pepper et al., 2000a). Also as yet unknown are which kind of mechanisms are involved in remodelling of mammary microvasculature; what difference it makes in PP compared to MP dairy goats; and whether having a normal dry period or not during the late gestation period might interfere with this remodelling of the mammary microvasculature.

In the following section, some of the factors that are believed to of crucial importance in the regulation of remodelling/angiogenesis and the function of mammary microvasculature, and which could potentially be involved in determining differences between CL and NL as well as PP and MP animals, are introduced.

VEGF family. Vascular endothelial growth factor, known to be the most potent factor in angiogenesis, possesses vascular permeability-inducing properties. Its receptor 1 regulates the mitogenic, and receptor 2 the permeabilizing, activity of VEGF, at the same time as receptor 1 has negative regulatory effects on receptor 2 (Conway et al., 2001). It seems from this that they might play an important role in mammary microvasculature remodelling, especially pre-partum, before initiation of a new lactation.

Angiopoietin family. ANGPT1 is involved in maturation of blood vessels; it regulates the formation and stabilization of the blood vessel network. ANGPT2 destabilizes the vasculature and thereby initiates angiogenesis in the presence of VEGF (Conway et al., 2001). Both ANGPT1 and ANGPT2 compete for their tie_2-receptor (RTK). They might play an important role not only in the initiation of angiogenesis especially around parturition time, but also have an impact on lactational performance by regulating the formation and stabilization of the mammary microvasculature.

Prostanoids. Cyclooxygenase 1 and 2 are believed to have role in angiogenesis, regulating arterio-venous differentiation, and contributing to the production of prostaglandins and thromboxane (Claria, 2003), potent vasoactive substances involved in local regulation of blood perfusion. Prostocylin I_2 synthase (PTGIS) regulates the synthesis of PGI_2, an arterial vasodilator; and thromboxane synthase regulates synthesis of TBXA, a venous vasoconstrictor that also affects vascular permeability (Claria, 2003). These two prostanoids might have role in regulation of mammary synthetic or secretory activity (Nielsen et al., 2004b) in addition to their role in regulation of vascular tone and blood perfusion.

Carbonic anhydrase. CA4 is believed to be the endothelial isoform of carbonic anhydrase, which catalyses the reversible hydration of CO_2 to HCO_3^- and H^+. It is positively correlated

with mammary gland synthetic activity in goats (Cvek et al., 1998), which suggests it could play a role in the sustenance of mammary secretory activity.

Milk synthesis

Mammary remodelling (capacity of the mammary gland) is important for lactation performance; and so is the extent to which this capacity can be exploited. In this respect, one needs to look at the provision of nutrients to the mammary gland. Nutrient supply for the synthesis of milk may affect milk production differently in early and later lactation when the capacity of the mammary gland has changed, and the priority of the mammary gland in the provision of nutrients relative to other tissues has also changed (see previous discussion). Nutrients are supplied to the mammary gland by the blood, from which they are taken up by MEC and converted into the milk constituents: lactose, fat and protein (fig. 3) (Madsen, 2002). Hence MEC is an important "biological factory", second in importance only to photosynthesis in sustaining mammalian life (Bauman et al., 2006; Patton, 1969).

In the following section, the processes involved in milk synthesis and the conversion of nutrients supplied to the gland into milk components, are discussed. The sensitivity of the mammary gland towards changes in nutrient provision during the course of lactation, is also considered.

Lactose synthesis. Lactose is a disaccharide composed of one glucose and one galactose unit, and is the dominant sugar of ruminant milk. Lactose synthesis is regulated by factors controlling the mammary gland synthetic capacity and therefore follows the general shape of the lactation curve (Uden and Danfaer, 2008). Lactose is the major osmotic factor in milk, determining the milk yield by controlling its volume (Knight et al., 1994; Riley et al., 2008). The major part of glucose taken up by the mammary gland (60-85 %) is converted to lactose in MEC of dairy cows and goats (Bickerstaffe et al., 1974; Nielsen and Jakobsen, 1993). However, it has been reported that in dairy goats mammary glucose uptake is related neither to long term nor to acute changes in the supply of glucose to the mammary gland (Nielsen et al., 2001). Rather, according to Bauman and Davis (1974), Kuhn (1983) and Madsen (2002), the availability of glucose in MEC for lactose synthesis depends on glucose transportation into the cell, as well as on its distribution in different pathways such as 1) lactose synthesis representing the most important fate, 2) the pentose phosphate cycle, providing NADPH for

de novo fatty acid synthesis, 3) glycolysis, providing glycerol-3-phosphate (which contributes in TG formation) and pyruvate (which contributes in ATP production); and finally 4) tricarboxylic acid cycle, providing alpha-keto acids for the formation of amino acids. The final step in lactose synthesis is catalysed by the enzyme complex lactose synthase, which consists of the catalytic subunit galactose transferase plus a modifying unit, the milk whey protein α-lactalbumin, which alters substrate affinity of galactosyl transferase to become capable of transferring a galactosyl group to free glucose and hence to create lactose (Akers, 2002). As previously mentioned, α-lactalbumin is up-regulated closely coordinated with the onset of lactation and is therefore a good marker of lactogenesis.

Fat synthesis. The main part of milk fat consists of triglyceride (TG; >95%) droplets which are coated with a milk fat globule membrane containing smaller amounts of phospholipids and cholesterol. In ruminants, there are over 400 different fatty acids secreted in milk fat TG, and the fatty acid composition depends on both the dietary manipulation of the animal as well as on the stage of lactation. The fatty acids incorporated into milk TG come from two major sources in ruminants (Akers, 2002; Jenness, 1974; Lock and Bauman, 2004): 1) direct uptake from TG and NEFA supplied by the blood, consisting mainly of long chain fatty acids (C16 and all ≥ 18 carbons), 2) *de novo* synthesis of short and medium chains (C4-C14) and part of 16 carbon (C16) fatty acids within the mammary gland from acetate (C2) and BHB (C4) taken up from the blood. Glucose via acetyl-coenzyme A on the other hand does not contribute to any significant extent to *de novo* fatty acid synthesis in ruminants, but only to the synthesis of the glycerol backbone. Lipids absorbed either from the digestive tract or synthesised in the liver are provided to the mammary gland in the form of lipoproteins (chylomicrons and very low density lipoproteins, respectively). Fatty acids can be released from these lipoproteins and be taken up by the mammary gland upon hydrolyses of TG by the mammary capillary wall enzyme, lipoprotein lipase (LPL), which converts TG to glycerol and fatty acids; these in turn can be taken up by the MEC (Lock and Bauman, 2004). NEFA originating from the body fat reserves are taken up by the MEC directly by passive diffusion. Plasma NEFA content, and hence energy balance, is thus highly correlated to milk fat content in milk (Chilliard et al., 2003). This explains why after parturition and in early lactation, when the animal is generally in a negative energy balance, milk fat content is high with a high proportion of C18:0 and C18:1 (Chilliard et al., 2003). When lactation peaks in dairy goats,

fat content drops (Chilliard et al., 1986; Mioc et al., 2008), the animal's feed intake increases, and the proportion of nutrients allocated for milk synthesis falls.

Protein synthesis. Milk has two major protein fractions (Akers, 2002; Ambrosoli et al., 1988; Bequette et al., 1998; Law and Brown, 1994): 1) Casein (α_s-, β-, κ- and γ-casein) (about 80% of milk proteins), a hydrophobic globular protein present in milk as a colloidal suspension, and 2) whey proteins or milk serum proteins (α-lactalbumin, β-lactoglobulin and proteose-peptones). Milk proteins are synthesized from amino acids derived from the blood supply. The ratio of mammary uptake of amino acid nitrogen compared to output of amino acid nitrogen in milk protein is about 1:1 (Bequette et al., 1996; Bickerstaffe et al., 1974; Guinard and Rulquin, 1994a; Guinard and Rulquin, 1994b; Madsen, 2002; Mepham, 1982). There is still however an excess uptake of certain essential amino acids (EAA) which in the mammary gland are used to synthesize nonessential amino acids (NEAA) (Akers, 2002). This indicates the role of EAA as amino group donors in transamination reactions where NEAA are synthesised *de novo* (Bequette et al., 1998; Madsen et al., 2005; Mepham, 1982). Generally (Bequette et al., 1998; Madsen, 2002; Mepham, 1982; Rudolph et al., 2007), intracellular amino acids contribute by: 1) being polymerised on ribosomes attached to the endoplasmic reticulum in MEC to form the milk protein (the most important fate), 2) synthesing the structural proteins and enzymes, and 3) producing ATP, NADPH and glycerol-3-phosphate via deamination to α-keto acids and oxidation processes in the citric acid cycle.

As previously described, when supply of other energy-yielding substrates is not increased, an increased supply of specific amino acids has been found to have a positive effect on milk protein production - in some situations (Hanigan et al., 2001; Rulquin et al., 1993; Weekes et al., 2006), but not all (Seymour et al., 1990). Milk protein synthesis in the mammary gland relies on energy in the form of ATP, and a shortage of nutrients to provide this energy supply may result in a poorer utilisation of amino acids supplied to the mammary gland. Acetate and glucose are the major energy suppliers to the mammary gland. Acetate is utilized in oxidative phosphorylation of adenosine nucleosides; this process generates predominantly ATP (Forsberg et al., 1984; Scott et al., 1976), in contrast to the situation where glucose is oxidized mainly through the pentose phosphate pathway to yield the NADPH required for *de novo* fatty acid synthesis (lipogenesis) (Chaiyabutr et al., 1980; Chaiyabutr et al., 2008). It is likely that supply to the mammary gland of energy in the form of ATP limits protein synthesis and hence the efficiency of the amino acids provided to the mammary gland. Conversely, it should be

theoretically possible to substitute amino acids in the diet to some extent and sustain milk protein synthesis by optimizing provision of energy-yielding substrates to provide extra ATP in the mammary gland, and at the same time improve the overall utilization of amino acids available to the animal. In an experiment with goats fed different levels of rumen-protected lysine and methionine, rather close correlations were observed between arterial concentrations for individual amino acids and the arterio-venous differences (AVD) across the mammary gland for these amino acids (Madsen et al., 2005). However, the magnitude of change in AVD over a given range of arterial concentrations was smaller in early as opposed to late lactation, indicating that the sensitivity of the mammary gland towards variations in amino acid supply may be different at different stages of the lactation process. Changes in mammary synthetic capacity during the course of lactation may determine to what extent amino acids may be substituted by energy-yielding (ATP) substrates without negative consequences for milk (protein) production.

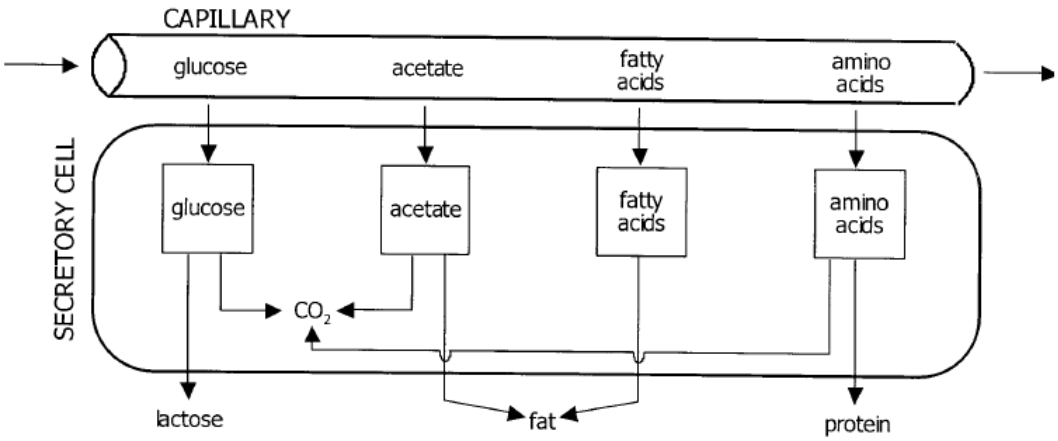


Figure 3- Simple diagram of conversion of nutrient uptake into milk components, modified from Cant et al. (2003; Madsen, 2002)

In conclusion, it remains to be established:

1) which factors are responsible for the changes in growth and remodelling of MEC and particularly the mammary microvasculature during gestation-lactation,

2) the role of the mammary microvasculature in determining lactation performance and persistency,

3) whether the factors which can account for different developmental patterns between PP and MP animals in early lactation, are also the factors responsible for the impaired mammary

(re)development in animals subjected to continued milking as opposed to animals allowed a normal dry period in late gestation,
4) whether changes in mammary synthetic capacity during lactation influence the extent to which amino acid supply to the mammary gland can be compensated by provision of energy-(ATP)-yielding substrates to sustain milk (protein) synthesis.

Knowledge on all these issues is important for future attempts to find new ways to improve lactation persistency and the overall lactation performance of dairy animals.

Methodology

It was aimed to: 1) determine the impact of parity number (PP vs MP) and dry period (CL vs NL) on mammary synthetic capacity through interference with cell turnover and function of MEC and microvasculature, and identify the involved regulatory factors, 2) Elucidate if milk (protein) synthesis is more sensitive towards variations in nutrient supply in the early compared to late lactating mammary gland, and whether provision of energy yielding substrates (acetate vs glucose) can compensate for an insufficient amino acid supply to the mammary gland and hence improve overall milk (protein) synthesis. These aims have been addressed in three experiments with dairy goats. The experiments were carried out at the experimental facilities either at Rørrendegård in Høje Taastrup or at Frederiksberg campus, Faculty of Life Sciences, University of Copenhagen, Denmark, and approved by the Danish Animal Experimentation Inspectorate, and complied with the Danish Ministry of Justice laws concerning animal experimentation and care for experimental animals. In the following after a short introduction of each experiment, the main methods and techniques used in those experiments are presented.

Experiment 1. Omitting the dry period: effects on mammary remodelling and metabolic activity (papers 1 and 2)

During years 2004-2006, nine dairy goats were used followed over one (5 goats) or two consecutive (4 goats) pregnancy-lactation periods. Goats were previously surgically prepared with exteriorized carotid arteries and milk veins. They were fed according to Danish requirements and milked manually (at 09:00 and 15:30) and fed twice a day (at 07:30 and 14:30), half the ration being given at each feeding. The experimental design was a randomized complete block design; the two udder halves in each animal were randomly assigned to two different treatments: one udder half was dried-off approximately 9 weeks pre-partum (normal lactation; **NL**), and the other udder half of the same goat was milked continuously (continuous lactation; **CL**) until parturition. Samplings were performed at different time-points during gestation and lactation: just before the NL gland was dried off (before dry period); within the first 2 weeks after drying-off (only NL glands); in the mid-dry period; within the last 2 weeks prior to parturition (late dry period); and at days 1 (day of parturition); 3; 10; 60; and 180 of lactation. At each sampling time, two blood samples were obtained from the exteriorized carotid artery and each of the milk veins after the afternoon

and following morning milkings, and mammary biopsies were obtained from each udder half after the morning milking. Half udder milk yields were recorded.

Experiment 2. Parity number: effects on mammary remodelling (paper 3)

The experiment was conducted during years 2004-2006. Mammary biopsies were obtained from both mammary glands of 3 primiparous (**PP**) and 6 multiparous (**MP**) (≥2 parity) dairy goats at parturition (d1), days 10, 60, and 180 of lactation. The 3 PP goats were followed in their subsequent 2nd lactation in the second year of the experiment. Feeding and milking was done as described for experiment 1.

Experiment 3. Continuous intravenous infusion of nutrient substrates: effects on mammary metabolic activity in early and late lactating dairy goats (paper 4)

This experiment was conducted in autumn 2007- early spring 2008 when goats were in late lactation (157±9 days postpartum) and in their following early lactation (21±1 days postpartum), respectively. At each stage, there was a pre-trial period of 4 weeks, where the goats became adapted to the less palatable parts of the diet, and to ensure equilibration of the rumen environment prior to initiation of the experimental treatments. The early and late lactating dairy goats were fed a basal diet deficient in energy (fulfilling 90% of requirements for NE) and protein (fulfilling 80% of requirements for Amino Acids Absorbable from the small intestine), according to the body weight and milk yields recorded in the last of the 4 pre-trial weeks, and based on the Danish feeding standards (Strudsholm et al., 1999), which are consistent with the nutrient requirements of dairy goats, as reported by Sauvant and Morand-Fehr (1989). Goats were randomly allocated to 4 treatments in a balanced 4 x 4 Latin Square design. The goats were milked and fed the restricted basal diet in two daily meals (at 07:00 and 17:00 h) during the first 3 days of each infusion period, and every 4 h for the last 24 h during the sampling period. The treatments consisted of 4 d (96 h) continuous intravenous infusions of isoosmotic solutions at pH 7.4 of saline as control, essential amino acids, sodium acetate and glucose with a 3 d rest period between each treatment. The essential amino acid, acetate and glucose infusions were designed to be isoenergetic calculated from the theoretical yield of ATP upon complete oxidation of the nutrients infused intravenously. Simultaneous arterio-venous blood samplings over each udder half (gland) were performed every 4 h during the last 24 h of infusion. Feed refusals from the last 24 hours of each infusion period were

collected. Milk yields were recorded at each milking, and proportionate milk samples were obtained from each milking during the last 24 h of each infusion period.

For both experiments 1 and 2, mammary morphology was characterised in biopsies by quantitative histology, and cell turnover was determined immunohistochemically (TUNEL and Ki-67). Transcription of genes encoding for factors involved in mammary epithelial cell (**MEC**) turnover and vascular function was quantified by quantitative reverse transcription PCR. In experiments 2 and 3, immediate determination of acid-base parameters was done on whole blood, and plasma was analyzed for selected metabolites and hormones. In the following, the main used methods and their general concepts are discussed.

Animal model

All experiments were conducted on dairy goats. Goats have often been used as experimental models for dairy cows in lactation studies due to their low cost and their similar selection for high milk production as dairy cattle (Anderson et al., 1981), but dairy goats appear to differ from dairy cows for instance in their ability to sustain extended and continuous lactation (omission of the dry period) (Fowler et al., 1991; Mackenzie, 1967). Observations from dairy goats may therefore not be directly applicable to the dairy cow and vice versa. Species comparisons are however fruitful as they can contribute to elucidate general physiological mechanisms underlying mammary function for instance in relation to lactation persistency.

Unilateral model. As suggested by Annen et al (2004), the udder halves within each animal can be used as individual experimental units. By such an approach, the animal can be used as its own control, since both udder halves within an animal have the same genetic heritage and are subjected to the same systemic regulatory factors such as nutrients and endocrine hormones, especially the circulating growth factors (Annen et al., 2004; Bachman and Schairer, 2003). Therefore, it makes it a reasonable approach to study local regulatory mechanisms within each udder half subjected to different treatments, and minimizes the number of experimental animals required compared to when the whole mammary gland between different animals are to be compared. It must, however, be considered that one udder half may have a certain impact on the function of the other (Annen et al., 2004; Capuco and Akers, 1999). Thus, when only one udder half is dried off, both milk yield (Hamann and Reichmuth, 1990; Henderson and Peaker, 1983), and growth (Capuco and Akers, 1990)

increases in a compensatory way in the lactating gland within the same udder, and the involution is partially inhibited in the gland which is not lactating (Akers and Heald, 1978; Akers and Keys, 1985). Pre-partum milking advances lactogenesis and milk production (Akers and Heald, 1978). While one gland is milked prepartum, this could possibly affect the nonlactating gland within the same udder, having a premature milk synthesis without removal, which might affect its subsequent lactation (Capuco and Akers, 1999). As Capuco and Akers suggested (1999), this could either result in a positive effect on the continuously milked gland or a negative effect on the dried gland within the same udder. However, the experimental approach with unilateral drying-off of one gland whilst continuously milking the other throughout the late gestation period, appears useful in the attempt to identify remodelling events responsible for determination of lactation performance in the subsequent lactation and identification of the potential key regulatory factors involved.

Biopsy technique. There have been a number of studies in dairy cows (Capuco et al., 1997), goats (Fowler et al., 1991) and sheep (Norgaard et al., 2008a), where animals were sacrificed at different time points during the pregnancy-lactation cycle to determine characteristics of the mammary gland. This is obviously the most correct way of truly assessing the total composition of the gland. But the main disadvantage of such an approach is the large inter-animal variation (Knight and Peaker, 1984), and the associated costs due to the need of high numbers of animals. Another disadvantage of this method is that the patterns of metabolic/cellular changes in the mammary gland can not be followed in the same animal over the course of time. Obtaining smaller biopsies of the mammary gland is an alternative approach, which has been used in several experiments with dairy cows (Miller et al., 2006), goats (Caja et al., 2006) and sheep (Norgaard et al., 2008a). This method (see appendix 1) allows samplings from the mammary gland of the same animal at different stages of pregnancy-lactation. Such a technique is preferred when changes within the mammary gland over the course of e.g. lactation are to be followed in the same animals, and the number of animals needed can be limited. The repeated biopsy sampling does apparently not affect milk production over a long-term period (Knight and Peaker, 1984). When obtaining small biopsies from a large organ like the ruminant udder, not all parts of the organs will have the same chance of being sampled, and therefore the biopsy samples may not be entirely representative of the whole organ. In experiments 1 and 2 we worked with adult goats which had experienced at least one parturition, and hence one complete period of mammary development

during pregnancy. The mammary gland in such animals can be assumed to be composed mainly of parenchymal tissue (Knight and Wilde, 1993), which was the main target of our study. Therefore, it should be possible by the biopsy procedure to get fairly representative samples of parenchymal tissue. The advantage of the mammary gland in general in this respect is that the tissue structures within the part of the gland that was sampled (see papers 1 and 3) have a fairly random orientation by nature and is composed of sphere like alveoli surrounded by the other tissue components. Hence, tissue structure in mammary biopsies obtained by our approach can be assumed to be fairly isotropic (randomly distributed), which is a requirement in unbiased stereology (Gundersen et al., 1988; Howard and Reed, 2005).

Histology

To be able to look into qualitative as well as quantitative changes of mammary tissue components, histological studies were performed (table 1, see also appendices 2 and 3). An interval of at least 30 μm between studied successive tissue sections was ensured so that individual cells were not recounted. Furthermore, a randomized direction of the sections on the slides was considered to make the further evaluations as unbiased as possible. The individual tissue sections were not numbered when cut, which would have made it possible for us to calculate the height of the same targeted object between two slides. Therefore, we were not able to calculate 3D volume density of the components we had in our tissue slides. Different duration of the staining steps, as well as different duration of being exposed to a fixative like ethanol, can affect the rate of tissue shrinkage. Every precaution was taken to ensure that all the samples were treated in the exact same way. For qualitative morphology, two staining methods, H&E and PAS, were chosen (briefly explained in appendices 4 and 5). When counting the different tissue fractions, stereological procedures were followed, using an unbiased counting frame, designed according to Gundersen et al. (1988).

Immunohistochemistry

To get an estimation of the quantity of MEC in mammary tissue, measuring the DNA content could be recommended (Capuco et al., 1997; Miller et al., 2006), but to get estimates of the relative ratios of cell proliferation and apoptosis, more specific measurements are required. Quantification of cell proliferation and apoptosis were done on mammary biopsies (papers 1 and 3) using well-known immunohistochemical labelling methods (Baldi et al., 2002; Fitzgerald et al., 2007; Norgaard et al., 2005) to identify cells undergoing cell proliferation

(labelling for Ki-67) and apoptosis (TUNEL method) (briefly explained in appendix 6). Slight modifications have been made in the protocols for Ki67 and TUNEL compared to procedures used in sheep and cow ((Norgaard et al., 2008a), which are mentioned in paper 3. In our caprine samples we observed a slightly stronger background colour and somehow even unspecific staining relative to bovine samples, especially with the TUNEL method, which can probably be ascribed to the chemical nature of bovine and caprine mammary tissue.

In the following (table 1), a general overview of the analytical steps performed on the paraffin embedded biopsy samples taken from the studied goat mammary glands, for both histological and immunohistochemical processes, are shown.

Table 1 - An overview of the processes performed on the paraffin embedded biopsy samples

	Purpose	Staining method	Sections	Pictures taken /section	Kind of grid	Counting points /grid	Counted points /sample
Paraffin embedded sample	Histology	H&E	2-3	3	100%	30 + Alveoli no.	~ 200 + Alveoli no.
		PAS	2-3	3	100%	30 + Alveoli no.	~ 200 + Alveoli no.
	Immuno-histochemistry	Ki-67	4	5	25%	all MECs	~ 700
		TUNEL	4	5	25%	all MECs	~ 700

Quantification of gene expression

Expression of genes was quantified in mammary biopsies for genes suggested to be important factors in regulation of apoptosis, cell proliferation, vascular function and remodelling in either the mammary gland or other tissues. This was done to study if these regulatory factors could account for the differences in mammary remodelling at the level of MEC and microvasculature in PP versus MP animals and between CL versus NL glands.

The candidate genes potentially involved in vascular function and angiogenesis/remodeling of the microvascular bed were: cyclooxygenase I (COX1), cyclooxygenase II (COX2), prostacycline synthase (PTGIS), thromboxane A2 synthase (TBXAS), carbonic anhydrase IV (CA4), angiopoietin I (ANGPT1), angiopoietin II (ANGPT2), tyrosine kinase tie2 receptor (RTK), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 1 (VEGFR1) and vascular endothelial growth factor receptor 2 (VEGFR2).

Candidate genes potentially involved in regulation of cell proliferation and apoptosis were: B-cell CLL/Lymphoma-2 (BCL2), Bcl2-Associated X Protein (BAX), cyclin D1 (CCND1), insulin-like growth factor I (IGF1), insulin-like growth factor I receptor (IGF1R), insulin-like

growth factor binding protein III (IGFBP3), insulin-like growth factor binding protein V (IGFBP5), transforming growth factor beta I (TGFB1), transforming growth factor beta I receptor I (TGFB1R1), transforming growth factor beta I receptor II (TGFB1R2), prolactin receptor (PRLR), lactoferrin (LTF), leptin (LPT) and leptin receptor (LPTR).
Alpha-lactalbumin (LALBA) was included as a candidate gene as an indicator of lactogenesis, as upregulation of LALBA synthesis is closely synchronized with the time of parturition and lactogenesis.
Transcription of target genes was quantified by real time RT-PCR (appendix 7). The oligonucleotide sequences for the genes were designed using the Primer Express software, version 2.0 (Applied Biosystems Inc, CA, USA) or Beacon Designer Version 7.00 software (Premier Biosoft International, CA, USA). All of the primer pairs and probes used in either of SYBR® or TaqMan® assays were designed based on the sequences available for goat (VEGF, VEGFR1 and VEGFR2), cow (COX1, COX2, PTGIS, TBXAS, CA4, ANGPT1, ANGPT2, RTK, CCND1, IGFBP3, IGFBP5, TGFB1, TGFB1R1, TGFB1R2, PRLR AND LTF) or sheep (BAX, BCL2, IGF1, IGF1R, LALBA, LPT and LPTR). The accession numbers, amplicon location and length, range of threshold cycle (CT) values in samples, and slope of standard curve as well as primer and probe sequences of the analyzed genes are presented in paper 1, tables 2 and 3.

Attempts were also made to look for changes in expression of the gene Relaxin receptor 1 (LGR7, leucine-rich repeat-containing G protein-coupled receptor) in the mammary gland. In rodents it has been reported that relaxin, along with other hormonal stimuli of pregnancy (mamogenenic hormones such as prolactin, growth hormone, estrogen, progesterone, and relaxin) could be associated with mammary development, especially around the time of parturition (Anderson et al., 1981; Harness and Anderson, 1977; Tucker, 1981). We were however not successful in finding proper primers and probes for this factor or its receptor 1 due to time limitation and the inadequacy of available primer sequences for goat.

All gene expression studies reported in the literature so far have been conducted on mammary biopsies consisting of a mix of different tissues and cell types present in the mammary gland. There is a risk that gene expression changes occurring in one particular cell type may not be detectable in such a mixed sample as discussed in more detail in papers 1 and 3. To really get an understanding of the regulatory events at the level of individual cell types, it would

therefore be necessary to study these individual cell types within the mammary tissue. For this reason in future studies, the use of laser microdissection is recommended to be able to work at the level of individual cell types among different mammary tissue components.

Blood sampling

To facilitate blood sampling and making it less stressful for the animals, we exteriorized the carotid arteries under the skin, and also made skin loops around both milk veins prior to the animals inclusion in the experiment. Brief description of surgical procedures according to Nielsen et al. (1995b) and catheterizations of *Vena Jugularis* are given in appendixes 8 to 10.

An important consideration when performing measurements of arterio-venous concentration differences (AVD) across the mammary gland is to obtain representative samples of arterial blood which supplies the mammary gland and of the venous blood draining this organ. The milk veins (*subcutaneous abdominal veins*) and pudic veins (*vena pudenda externa*) are the quantitatively important veins draining the udder, and the valves in these veins become incompetent during the course of 1st lactation in ruminants, i.e. can transport blood in either direction. During sampling of blood from the milk veins, it is therefore essential to clamp the pudic vein to avoid contamination of mammary blood with blood from non-mammary origin (Linzell, 1960). Except from superficial arteries supplying extremities, blood samples from any artery in the body can be regarded as representative with respect to nutrient composition. With the exception of blood gasses, the majority of nutrients have similar concentrations in the carotid artery as in the jugular vein, and jugular vein blood can therefore also be sampled instead of arterial blood when only concentrations of major nutrients are of interest. Arterial concentration (Miller et al., 1991) as well as rate of mammary blood flow (Madsen et al., 2005; Madsen et al., 2008) could both affect mammary uptake and AVD for different nutrients.

As described in papers 2 and 4, blood samples were collected in 1 ml LiHep coated syringes and immediately analyzed for acid-base parameters such as pH, $HCO3^-$, O_2 and CO_2 content which are involved in vascular function and hence the function of the milk synthesizing cells. Other blood samples were collected in 10 ml vacutainers coated with EDTA, and stored on ice till centrifugation (3000 gav at 4 °C for 12 min). Plasma was transferred to labeled cryo-tubes, and were analyzed for contents of metabolites involved in synthesis of the major milk components (glucose, acetate, beta-hydroxy-butyrate (**BHB**), non-esterified fatty acids

(**NEFA**), triglycerol (**TG**)), urea, and metabolic hormones (IGF1, insulin) using commercially available kits (described in further details in paper 4).

Continuous intravenous infusions (see also appendix 10)**.** In the continuous infusion trial, goats were fed a basal diet (see paper 4, table 2) designed to meet approximately 90% and 80% of daily requirements for energy and amino acids absorbable from the small intestine, respectively, according to their body weights and milk production in the two stages of lactation, early and late. On top of the daily basal diet, goats were given continuous intravenous infusions of one of 4 isoosmotic solutions at pH 7.4 (960 ml/d for 4 days) containing either saline as control, glucose, sodium acetate, or a mixture of essential amino acids composed to match the relative proportion of essential amino acids (**EAA**) in milk protein determined in a previous experiment with this goat breed (Madsen et al., 2005). There is no simple relation between what is fed to a ruminant animal and the absorption of EAA and glucose (Danfaer et al., 1995; Seymour et al., 1990) due to rumen fermentation. To provide a certain and known amount of a given nutrient, it has to be provided after the level of the forestomach fermentation, and in this experimental approach providing nutrients via intravenous (systemic) infusions was preferred. The EAA infusion was designed so that the total supply of amino acids (absorbed + infused) reached 105% of requirements for amino acids absorbable from the small intestine, and glucose and acetate infusions were calculated to be isoenergetic with the EAA infusion based on the potential yield of ATP from oxidation of the infused nutrients in the body. Composition of the infusates are shown in paper 4, table 1. Each treatment period lasted one week, where the intravenous infusions were initiated at 12:00 on day 1 (Monday) and concluded at 12:00 on day 5 (Friday), followed by a 3 day rest period in between. Samplings were performed during the last 24 hours of each infusion period.

Brief summary of papers

List of included papers

Paper I:

Continuous Lactation Effects on Mammary Remodelling During Late Gestation and Lactation in Dairy Goats. S. Safayi, P. K. Theil, L. Hou, M. Engbæk, J. V. Nørgaard, K. Sejrsen, M. O. Nielsen, Journal of Dairy Science, *In press (doi:10.3168/jds.2009-2507).*

Paper II:

Continuous Lactation Effects on Mammary Extraction Rates of Nutrients in Dairy Goats. S. Safayi, M. O. Nielsen, Pages 652-653 in Ruminant physiology. Digestion, metabolism, and effects of nutrition on reproduction and welfare (eds. Y Chilliard, F Glasser, Y Faulconnier, F Bocquier, I Veissier and M Doreau), 2009. Wageningen Academic Publishers, Wageningen, The Netherlands.

Paper III:

Mammary Remodelling in Primiparous and Multiparous Dairy Goats During Lactation. S. Safayi, P. K. Theil, V. S. Elbrønd, L. Hou, M. Engbæk, J. V. Nørgaard, K. Sejrsen, M. O. Nielsen, Journal of Dairy Science, *Accepted by Journal of Dairy Science (JDS-09-2422.R2).*

Paper IV:

Intravenous Supplementation of Acetate, Glucose or Essential Amino Acids to an Energy and Protein Deficient Diet in Early and Late Lactating Dairy Goats: Effects on Milk Production and Mammary Nutrient Extraction. S. Safayi, M. O. Nielsen, Journal of Dairy Science, *To be submitted to Journal of Dairy Science.*

Paper I & II:

Continuous Lactation Effects on Mammary Remodelling and Mammary Extraction Rates of Nutrients during Late Gestation and Lactation in Dairy Goats

Objectives

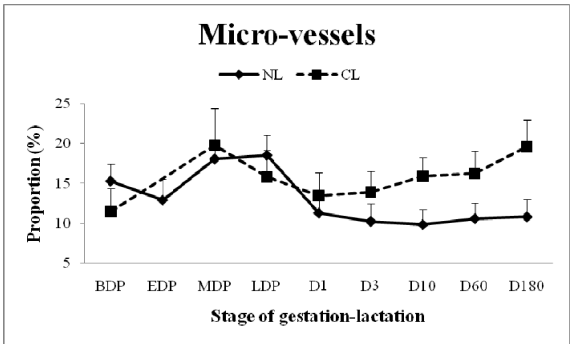
In these two papers we aimed to: 1) elucidate whether continuous milking during late gestation in dairy goats negatively impacts mammary remodelling and hence milk production in the following lactation, 2) identify the regulatory factors responsible for changes in cell turnover and angiogenesis in the continuously lactating mammary gland, and 3) determine if continuous lactation in the dairy goat will increase the efficiency of nutrient extraction across the mammary gland in the following lactation.

Material and methods

Nine dairy goats were followed over one (5 goats) or two consecutive (4 goats) pregnancy-lactation periods. Goats were previously surgically prepared with exteriorized carotid arteries and milk veins. They were milked manually (at 09:00 and 15:30) and fed twice a day (at 07:30 and 14:30), half the ration being given at each feeding. The experimental design was a randomized complete block design; one udder half was dried-off approximately 9 weeks pre-partum (normal lactation; NL), and the other udder half of the same goat was milked continuously (continuous lactation; CL) until parturition. After morning and afternoon milking, two blood samples were obtained from the exteriorized carotid artery and each of both milk veins, and mammary biopsies were obtained from each udder half just before the NL gland was dried off (BDP; before dry period), within the first 2 weeks after drying-off (EDP; early dry period), in the mid-dry period (MDP), within the last 2 weeks prior to parturition (LDP, late dry period), and at days 1 (the day of parturition), 3, 10, 60, and 180 of lactation. Mammary morphology was characterised in biopsies by quantitative histology, and cell turnover was determined by immunohistochemistry (TUNEL and Ki-67). Transcription of genes encoding factors involved in mammary epithelial cell (MEC) turnover and vascular function was quantified by quantitative reverse transcription PCR. Blood samples were taken for immediate determination of acid-base parameters, and plasma was analysed for glucose, non-esterified fatty acids (NEFA), acetate, beta-hydroxy-butyrate (BOHB), triglycerol (TG), and urea. Mammary extraction (E) rates of metabolites were calculated as mammary arterial-milk vein concentration difference (AVD) divided by arterial concentration.

Main results

- We experienced considerable problems in preventing the glands from drying off spontaneously in very late lactation.
- Milk yield turned out to be virtually unaffected by CL in the glands that were capable of completing a continuous lactation.
- CL glands had a 3-7% lower proportion of MEC in the subsequent mid-late lactation compared to NL glands, due to lower proliferation rate during MDP-LDP.
- At the time of parturition (and throughout lactation), the mammary glands subjected to CL had smaller alveoli, more fully differentiated MEC and a substantially larger capillary fraction compared with NL glands. None of the studied genomic factors could account for these treatment differences.



- MEC undergoing proliferation occurred predominantly in the intermediately differentiated rather than in the poorly and fully differentiated MEC (69%, 23% and 9%, respectively). We found no apoptotic cells among poorly differentiated MEC, but only in the intermediately or fully differentiated MEC.
- The higher rate of apoptosis in CL glands at d3 coincided with a drop in the proportion of fully differentiated MEC, which was not observed in NL glands.
- AVD and/or E differed for some metabolites (glucose, TG, LCFA and BHB) either significantly or numerically between the CL and NL glands during the late gestation period. This could reflect differences in extraction activity between the CL glands that remained lactating and the NL glands that were dried off prepartum.
- There was no significant impact of the prepartum milking of the CL glands on efficiency of mammary nutrient extraction in the subsequent lactation

Conclusions and perspectives

CL suppressed MEC renewal, due to suppression of pre-partum cell proliferation; however, the rate of apoptosis was unaffected. Continuously lactating mammary glands of goats therefore entered the subsequent lactation with a smaller MEC population, as known in dairy cows. CL glands throughout lactation had a larger micro-vessel proportion, lower proportion of MEC, but more fully differentiated and likely older population of MEC compared to NL glands. If fully differentiated MECs have higher secretory activity, this could in part explain why goats can lactate continuously without major negative impact on milk yield, in spite of the smaller population of MEC. But, the observation that extractions of nutrients were unaffected by CL do not lend immediate support to the hypothesis that these more fully differentiated MECs could have a more efficient nutrient extraction reflecting a higher metabolic activity in the subsequent lactation.

We were not able to resolve why continued milking during the late gestation period interferes with MEC and vascular remodelling within the mammary gland, and what regulatory mechanisms are responsible. So two puzzling questions remain: Why is the full restoration of the MEC population and overall mammary remodelling inhibited just because of continuous milk removal from a mammary gland throughout gestation? And why is this associated with a marked depression in milk yield in dairy cows in the subsequent lactation, but apparently not in the dairy goat that (with some difficulty) manage to lactate continuously?

Paper III:

Mammary Remodelling in Primiparous and Multiparous Dairy Goats During Lactation

Objectives

The overall aims of this study were to elucidate whether differences in lactational performance between primiparous (PP) and multiparous (MP) mammary glands are related to the time course of development and maintenance, not only of the MEC population but also of the mammary vasculature which sustains synthetic activity. We tried to identify the underlying regulatory factors or mechanisms responsible for developmental differences between PP and MP mammary glands. We aimed to address these issues in a study in dairy goats and to the extent possible make comparisons to what is known in the dairy cow.

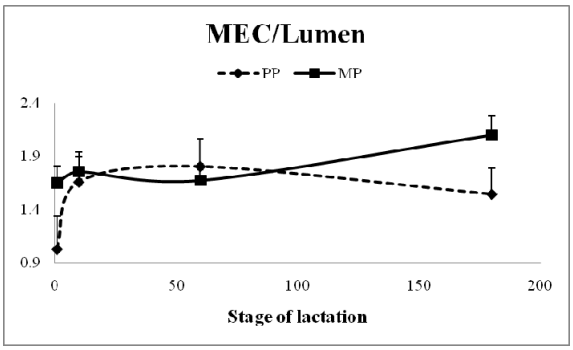
Material and methods

In this study, mammary biopsies were obtained from both mammary glands of 3 PP and 6 MP (≥2 parity) dairy goats at parturition (d1), days 10, 60, and 180 of lactation. Performing real time RT-PCR, quantitative histology (PAS and H&E) and immunohistochemistry (TUNEL and Ki-67) on the biopsies, we determined MEC turnover and differentiation, changes in morphology of the micro-vascular system, and the changes in patterns of expression of the major factors responsible for regulation of mammary cell turn-over as well as vascular function and angiogenesis.

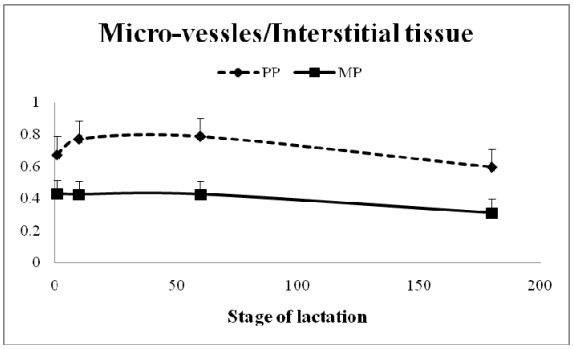
Main results

- Mammary glands of PP goats compared to of MP goats:
- were less compositionally differentiated at parturition
- had higher rates of MEC cell proliferation in early lactation
- had similar high rates of apoptosis right after parturition, which decreased to almost zero in PP glands over the course of lactation and numerically lower than in MP glands in mid-late lactation.
- had higher MEC survival and differentiation throughout lactation

- had substantially lower MEC:lumen ratio (1.0:1 and 1.6:1, respectively) at the time of parturition, but by d 60 of lactation the ratio in PP glands (1.8:1) had increased to the same level as in MP glands (1.7:1).



- showed lower expression of angiogenic factors (VEGFR1, VEGFR2, ANGPT1, ANGPT2 and RTK) as well as PRLR and LALBA at the time of parturition, but this had reversed by d10, where highest expression levels were found in PP glands.
- Had markedly higher micro-vessel to interstitial tissue ratio (71% and 41%, respectively) during the whole lactation period. This ratio decreased in parallel in PP and MP glands from early lactation and reached the lowest levels in late lactation in both PP and MP glands (59% and 31%, respectively).



- had higher expression of number of genes (COX1, COX2, PTGIS and TBXAS) encoding factors regulating vascular tone and function, especially in late lactation.

- Parity significantly impacted expression of the majority of genes involved in regulation of cell turnover and lactogenesis (BCL2, IGF1, IGFBP3, IGFBP5, TGFB1, TGFB1R2, PRLR, LALBA, LPT and LPTR), but not BAX, CCND1, IGF1R, TGFB1R1 and LTF.
- Expression of the antiapoptotic factor BCL2 corresponded with the high rates of apoptosis at parturition and the higher apoptotic rates in PP compared to MP glands.

- expression of IGF1, LPT and LPTR were at similar levels in MP and PP glands at the time of parturition, but with progress of lactation, expression of these factors increased in PP glands, which was in contrast to MP glands where expression levels remained constant or even diminished. This coincided with the more pronounced shift from poorly over intermediate to fully differentiated MEC in PP compared to MP glands.
- IGF1, LPT and LPTR exhibited the same pattern of expression changes as COX1, COX2, PTGIS and TBXAS.

Conclusions and perspectives

We have found that initiation of lactation and growth of the mammary gland continues further into early lactation in the PP compared to the MP dairy goats. This not only applies to MEC renewal, but also to development of vascular function (angiogenesis). This combined with improved MEC survival and maintenance of vascular competence, could explain the reported higher lactation persistency in PP compared to MP ruminants. There is an orchestrated change taking place to regulate the MEC and vascular function during normal lactation and to ensure coordination of function in these two important tissue components of the mammary gland. We have provided evidence that the set of genes regulating these orchestrated changes, are also basically the ones which can explain the different patterns of mammary development and performance during lactation between PP and MP animals, and hence the factors encoded for by these genes must be important determinants of lactational persistency. We therefore suggested that development and function of the mammary microvasculature may be just as essential for the overall integrity of the mammary gland and lactational persistency as the MEC. This issue should be addressed in future studies.

Paper IV:

Intravenous Supplementation of Acetate, Glucose or Essential Amino Acids to an Energy and Protein Deficient Diet in Early and Late Lactating Dairy Goats: Effects on Milk Production and Mammary Nutrient Extraction

Objectives

The aims of the present project were to determine whether : 1) provision of energy (ATP) yielding substrates can compensate for an insufficient amino acid supply to the mammary gland and hence improve overall utilization of amino acids for milk protein synthesis, 2) acetate is more efficient than glucose in stimulating milk synthesis and particularly its milk protein content; and 3) milk synthesis is more sensitive towards changes in nutrient provision in early lactation (**EL**) compared with late lactation (**LL**).

Material and methods

Four dairy goats used in this study were previously surgically prepared with exteriorized carotid arteries and milk veins. They were fed a basal diet deficient in energy (90% of requirements) and protein (80% of requirements), and were randomly allocated to 4 treatments in a balanced 4 x 4 Latin Square design. The treatments consisted of 4-d continuous intravenous infusions of isoosmotic isoenergetic solutions of essential amino acids (**EAA**), sodium acetate (**ACE**) and glucose (**GLU**) with saline (**SAL**) as control. There was a 3-d rest period between each treatment. Simultaneous arterio-venous blood samplings over each udder half (gland) were performed every 4 h during the last 24 h of infusion. Blood acid-base parameters and plasma concentrations of glucose, non-esterified fatty acids, beta-hydroxy-butyrate, triacylglycerol and urea were determined. Mammary extraction (**E**) rates of metabolites were calculated as mammary arterial-milk vein concentration difference (**AVD**) divided by arterial concentration. Milk production over the last 48 h of infusion was recorded, and milk fat and protein contents determined.

Main results

- Milk yield and energy corrected milk, milk fat and protein yields in g/last 48 hours were significantly higher in EL than LL.
- Gross milk yield was significantly higher on GLU and EAA treatments in EL compared to the SAL control treatment, and yield of ECM was increased on ACE relative to SAL. In

LL, nutrient supplementations did not affect gross milk yield, but ECM was increased on EAA compared to other treatments.

- Protein percentage was lowest on GLU compared to other treatments, and protein yields were increased on EAA and ACE, but not on GLU compared to SAL in EL. In LL, milk protein percentage was higher on EAA than GLU and milk protein yield was higher on EAA compared to ACE and GLU.
- Fat percentages were decreased by GLU and EAA compared to ACE and SAL in EL, and highest fat yield in EL was observed on ACE. In LL, GLU also lowered milk fat percentages whereas EAA increased in contrary to the effect in EL.

Conclusions and perspectives

An insufficient amino acid supply to the mammary gland of dairy goats can be compensated in EL but perhaps not LL by increased mammary supply and uptake of an energy yielding substrates, which specifically contributes to ATP formation in MEC. The mammary gland may thus be relatively less sensitive towards variations in amino acid supply in EL compared to LL. This suggests there might be scope for development of differential protein recommendations for ruminants in early and late lactation, and this issue should be pursued in future studies.

Discussion

Three experiments have been carried out with dairy goats to elucidate: 1) the impact of parity number (PP vs MP) and dry period (CL vs NL) on mammary synthetic capacity through interference with cell turnover and function of MEC and microvasculature, and identifying the regulatory factors; and 2) the sensitivity of the mammary gland in early compared to late lactation towards variations in nutrient supply, and whether provision of energy yielding substrates (acetate vs glucose) can compensate for an insufficient amino acid supply to the mammary gland and hence improve overall milk (protein) synthesis.

Parity and omission of the dry period in late gestation impact lactation performance due to similar patterns of changes in mammary growth and remodelling

PP vs MP. Our results confirmed recent studies in cow (Norgaard et al., 2005), sheep (Norgaard et al., 2008a) and goat (Caja et al., 2006) that mammary (re)development in MP goats takes place predominantly in late pregnancy with only little occurring after parturition. In contrast, a continued growth into lactation in PP mammary glands has been seen in our studies as also reported by other dairy goats (Anderson et al., 1981; Knight and Peaker, 1984) and dairy cows (Miller et al., 2006). Our results indicate that PP glands are less compositionally differentiated at parturition compared to MP-NL glands. Thus it seems that MEC proliferation as well as differentiation continues further into lactation in PP compared to MP goats, and the first hypothesis raised in the "Introduction" could thus be confirmed.

Our results on the proportional changes of poorly, intermediately and fully differentiated MEC during gestation-lactation period and previous reports in Holstein cows (Akers et al., 2006) make it reasonable to suggest that the poorly differentiated MEC are the newly formed cells in the mammary gland, that need to differentiate further to acquire full lactational ability. In our studies, the proportion of intermediately differentiated MEC decreased over the course of lactation in PP glands, whilst the proportion of fully differentiated MEC increased, especially from d10 and onwards. These changes most likely reflect that intermediately differentiated MEC progressively become differentiated into fully differentiated MEC in PP glands. As a consequence of lower rate of apoptosis from d10 and onwards in PP compared to MP glands, this would overall indicate that the MEC population in PP glands in addition to having new cells added during lactation, also were more persistent and hence capable of remaining lactating for a longer period of time.

The micro-vessel to interstitial tissue ratio in MP changed in parallel but at a lower level in PP glands over the course of lactation, which presumably reflects microvasculature regression, consistent with the changes observed over the course of lactation in rats (Yasugi et al., 1989). It has been suggested in mouse, that microvascular regression either follows the alveolar degeneration (Matsumoto et al., 1992) or could be correlated to, or even be a consequence of, MEC involution (Djonov et al., 2001). It has not been definitively proven what comes first, but it appears evident that the function and integrity of the MEC is dependent on and closely coordinated with the vascular system responsible for provision of nutrients and removal of waste products, essential to sustain MEC metabolism.

NL vs CL in multiparous goats. Despite the negative impact of CL on MEC renewal in MP goats, milk yield turned out to be virtually unaffected by CL in the glands that were capable of completing a continuous lactation. CL glands had a higher proportion of more fully differentiated MECs. But there was no significant impact of the pre-partum milking of the CL glands on efficiency of mammary nutrient extraction in the subsequent lactation, which does not lend immediate support to the hypothesis that the more fully differentiated MECs in the CL compared to NL glands would have a more efficient nutrient extraction reflecting a higher metabolic activity. It would therefore be relevant to find out how regulation of mammary remodelling differs between cows and goats and thus find the explanation why the milk yield in the subsequent lactation in goats subjected to CL is not negatively affected to the extent observed in dairy cows. The CL mammary gland in both species (Capuco and Akers, 1999; and Paper 1) apparently possesses MEC in a stage of differentiation capable of undergoing both cell proliferation and responding to endocrine factors initiating lactogenesis just as in NL glands. We can speculate that the MEC in goats may survive longer than in cows, but it remains to be established if it is so.

CL resulted in a larger ratio of capillaries-to-alveoli during lactation compared to NL glands,, which could be indicative of a greater blood supply to the MEC and favour exchange of nutrients and waste products across the capillary-MEC barrier in those glands. This could be another explanation why milk yield in goats is unaffected by CL, despite the negative impact of CL on pre-partum cell renewal. The capillary-to-alveolar ratio will normally be at its lowest in early lactation and increase as lactation progresses, reflecting a high number of very small capillaries surrounding large alveoli in the beginning of lactation, and increasingly larger and fewer capillaries surrounding alveoli as lactation progresses (Nielsen MO et al., unpublished data, and present study). This ratio is thus inversely related to mammary blood

flow changes (Nielsen et al., 1990) and efficiency of nutrient extraction in the mammary gland during lactation (Nielsen et al., 2001), very small capillaries would favour a more efficient nutrient exchange across the capillary – MEC barrier due to smaller diffusion distances, and extraction rates were in fact higher in EL compared to LL (Madsen et al., 2008; Nielsen et al., 2001 and paper 4). In our study, there seemed to be an increased contact between microvasculature and alveoli with progressing lactation, which could be a consequence of either the enlargement of capillaries (Nielsen MO et. al., unpublished data) or more meandering (Djonov et al., 2001; Matsumoto et al., 1992). This was more evident in CL than NL glands. Our findings therefore indicate that CL glands enter the new lactation with an older population of MEC surrounded by a similarly older (and possibly more developed) capillary network. As a result, nutrient exchange across the capillary-MEC barrier would occur less efficiently, but the decrease in vascular resistance (larger capillary diameter) could possibly allow for a compensatory increase in mammary blood flow. Future studies will need to be conducted to reveal if milk yield in this way could be maintained relatively unaffected by CL in the goat due to a higher mammary blood perfusion per unit of milk synthesized.

Altogether, similar to the effects of parity, it is also evident that CL would affect mammary remodelling not only by influencing MEC turnover and differentiation, but also MEC activity which appears to be in close coordination with the surrounding microvasculature, and thereby the overall mammary gland function. Improving our understanding of these remodelling events in the mammary gland in future studies are required to be able to optimise mammary gland performance and hence production capabilities of dairy animals.

Omission of the dry period in late gestation interferes with mammary remodelling through regulatory factors that are not identical to the factors responsible for differential mammary development in PP versus MP goats

Mammary epithelial cells. Initiation of lactation in MP ruminants is associated with up-regulation of the PRLR in MEC (Wall et al., 2006), and initiation of copious milk secretion including the whey protein LALBA (Forsyth and Neville, 2009; McFadden et al., 1987). In this study expression of these factors was therefore at its highest at the time of parturition in MP goats. However, in PP goats both PRLR and LALBA expression increased from parturition to peak at d10, which is yet another indication that initiation of lactation and growth in the PP mammary gland continues further into early lactation than in MP, and that initiation of lactation possibly is a delayed or prolonged process in the PP dairy goat. Theil et

al. (2006) showed that expression of PRLR in mammary glands of sows is crucial in order to ensure continued growth of the mammary gland at parturition and to initiate lactation. The PRLR could therefore be one of the overall factors involved in coordinating mammary growth, function and angiogenesis in the PP dairy goat, and the delayed lactogenesis and continued development of the PP mammary gland into lactation could be a result of sustained expression of the PRLR. The underlying reason for this differential expression pattern in PP and MP goats' mammary glands is not known.

We were unable to relate differences in rates of apoptosis during the lactation period or between PP and MP glands to expression of BAX, which is an apoptotic factor (Reed, 1998). Our results suggest that the antiapoptotic factor BCL2 plays a greater role than BAX in regulation of apoptosis from parturition and during lactation. It can, however, not be ruled out that more cell specific gene expression studies could reveal that BAX may be involved in this regulation of MEC turnover as well.

Insulin-like growth factor 1 has been reported to stimulate MEC growth, proliferation and cell survival (Knight, 2000), and LPT has been reported to be involved in regulation of MEC proliferation and/or differentiation prior to parturition (Chilliard et al., 2001). We found that expression of IGF1, LPT and LPTR were at similar levels in the MP and PP mammary gland at the time of parturition, but with progressing lactation, expression of these factors increased in PP glands, whereas expression levels remained constant or even diminished in the MP mammary glands. This coincided with the more pronounced shift from poorly over intermediately to fully differentiated MEC in PP compared to MP glands. It is therefore tempting to speculate that IGF-1 and LPT are important factors involved in regulating differentiation and cell survival during lactation in general, and also contributing to explain the different developmental patterns between PP and MP glands over the course of lactation.

The regulatory factors determining development and differentiation of MEC during the normal lactation in the mammary gland of MP goats thus seem to be the same set of factors responsible for the differential pattern of development in the PP ones. It would be valuable to gain insight into the underlying reason for the impact of parity on expression of this whole range of MEC genes, as this is a key to understanding aspects of cell survival, metabolic activity and hence lactation persistency.

We were not able to demonstrate any significant effects of CL on expression of any of the genes encoding for factors with reported effects on cell turnover and differentiation. Therefore, the factors or mechanisms explaining the impact of CL in late gestation on cell

renewal and differentiation still remain to be identified, and may apparently be different from the factors involved in the normal regulation of mammary remodelling during gestation and lactation. However, we can not rule out that our failure to link the cell turnover events to changes in gene transcription in MEC in our mammary biopsies could have been "masked" by the presence of several other cell types. Obviously the impact of continued milking in late gestation on cell renewal and mammary remodelling must be mediated by local mechanisms in the gland subjected to CL, since it is possible to continuously milk one udder half in the animal whilst drying-off the other.

Mammary micro-vasculature. This is the first report of quantitative gene expression analysis for factors involved in angiogenesis and vascular remodelling (VEGF, VEGFR1, VEGFR2, ANGPT1, ANGPT2 and RTK) in the mammary gland of ruminants to our knowledge, and only few studies in rodents have looked into the mammary vasculature and regulation of its remodelling (Djonov et al., 2001; Pepper et al., 2000b). Parity appeared to influence the whole range of studied genes associated with vascular function and angiogenesis in our dairy goats. VEGFR1, VEGFR2, ANGPT1, ANGPT2 and RTK are all angiogenic factors (Shibuya, 2001; Thurston, 2003), and these genes as well as PRLR and LALBA were generally higher expressed in MP compared to PP at parturition, but this had reversed by d10, where highest expression levels were found in PP glands. It has been suggested that the MEC is the main source of angiogenic factors in the mammary gland suggested by Pepper et al. (2000b), and as such it appears logical that expression of angiogenic factors would follow the developmental pattern of MEC described above during lactation and in mammary glands of different parity. Primiparous glands thus continue to develop also their vascular system post-partum to a greater extent than in MP goats. Furthermore, we observed that a number of other genes (COX1, COX2, PTGIS and TBXAS) encoding for factors regulating vascular tone and function (Nielsen et al., 1995a) were higher expressed in PP than MP glands, especially in late lactation. These factors are synthesized within the capillary endothelial cells in addition to MEC. Cyclooxygenase (COX1 and COX2) are key enzymes involved in synthesis of a number of vasoactive substances (Simmons et al., 2004), and the enzyme PTGIS specifically regulates the synthesis of prostacyclin (PGI2), which has been reported to be a potent vasodilator in the mammary gland (Nielsen et al., 2004a). Higher expression of genes encoding for potent vasodilators could likely be associated with improved blood perfusion in PP glands in more advanced stages of lactation, and would thus contribute to explain why lactation persistency may be higher in PP goats. The reason for higher expression of these

genes encoding for factors with vasoactive action is not known. It is noteworthy that IGF1, LPT and LPTR exhibited the same pattern of expression changes as the vasoactive factors COX1, COX2, PTGIS and TBXAS. This could suggest that the differential expression of the vascular function genes was linked to the differential development of the MEC in PP compared to MP glands, and in this way coupled to differential development also of the mammary vascular system. An important message thus seems to be that the changes in vascular and MEC function appear to be regulated in a coordinated manner. Our studies give rise to the question whether the vascular system may be involved in determining lactation persistency, and this issue deserves to receive more attention in the future, as so little work has been carried out in this area so far.

As mentioned above, CL did not affect expression of any of the genes encoding for MEC turnover, but CL did affect expression during gestation and at the day of parturition for some of the genes encoding for factors regulating vascular function. The higher gene expression of CA4 ($P<0.06$) particularly in the pre-partum period and of VEGFR1 during the first part of the dry period in CL glands may be ascribed to the fact that those glands had a MEC population that was prevented from involution, and thus maintained in lactation. Consequently a functional capillary network was sustained as well. Statistically or numerically higher expressions of ANGPT1, VEGF, VEGFR1, VEGFR2 and COX2 in CL glands at d1, suggest that the CL glands experiences a higher rate of vascular growth than the NL glands around parturition, probably renewing part of the ageing vascular system at this time, which, in the NL gland would have happened prior to parturition in parallel to the renewal of the MEC population.

Altogether, the same regulatory factors appear to be involved in vascular remodelling irrespective of whether this is a result of parity or induced by CL, whereas CL will affect MEC turnover by different mechanisms than the ones that regulate MEC turnover during the normal course of lactation in PP and MP animals. The second hypothesis could therefore only partly be confirmed. Identification of such mechanisms in future studies may lead to a better understanding of how lactation performance and persistency can be favoured in dairy animals.

Milk protein synthesis in the lactating mammary gland is less sensitive towards variations in amino acid supply in early compared to late lactation provided the energy supply is adequate

It is commonly believed that nutrient supply to the mammary gland is the main limiting factor for milk production in EL, whereas synthetic capacity becomes limiting in LL, as MEC die and feed intake increases to the extent where nutrient supply no longer limits nutrient uptake in the mammary gland (Madsen et al., 2005; Wilde and Knight, 1989). In LL, the population of transporters in the MEC for both glucose (Shennan and Peaker, 2000) and amino acids (Sharma and Kansal, 2000) decrease compared to EL. The same has been reported for activity of the capillary enzyme lipoprotein lipase involved in mammary uptake of plasma TG (Neville and Picciano, 1997). Hence more nutrients provided by the blood will escape mammary uptake. Consequently AVD and E were significantly lower in LL compared to EL, reflecting the decreased synthetic capacity and metabolic activity in the organ, with a decreasing capability to extract nutrients from whatever volume of blood that flows through it. Even for nutrients where concentration gradients are believed to be the driving force for mammary uptake (acetate, BOHB and NEFA), efficiency of mammary uptake decreased as lactation progressed, showing that extraction of such nutrients is determined not only by arterial concentrations, but probably also by the intracellular concentration and hence metabolic activity in the mammary epithelial cells. With progressing lactation, it therefore appears logical to assume that milk synthesis within the mammary gland should become less sensitive to changes in supply of nutrients. In LL, additional supply of acetate and glucose did indeed fail to drive up milk synthesis, although AVD for acetate increased on ACE compared to all other treatments. This indicates that mammary gland synthetic capacity was the main limiting factor for milk synthesis at this stage of lactation and not substrate supply. However, in contrast to ACE and GLU, supplementation of additional EAA to the basal energy and protein deficient diet could drive up synthesis of ECM, milk protein as well as fat in LL.

In a previous goat experiment we showed that AVD for lysine and methionine across the mammary gland increased linearly with A in both EL and LL in response to increased dietary supply. But it required higher arterial concentrations to drive a given uptake (AVD) in LL (Madsen et al., 2005). It has been shown by Sharma and Kansal (2000) in the rabbit that the relative abundance of different amino acid transporters in the mammary epithelial cell membrane changes during lactation. This observation along with our results, suggest that in contrast to our third hypothesis, the mammary gland in EL may in fact be more robust

towards changes in amino acid supply, capable of maintaining amino acid uptake even at rather low arterial concentrations. And this may explain why an insufficient amino acid supply in EL could be compensated by a larger energy (ATP) supply, whereas this was not possible in LL.

Deficiencies in dietary provision of protein can be compensated in EL but not LL by provision of energy (ATP) yielding substrates to sustain milk (protein) synthesis

In agreement with a study done on dairy cows by Cant et al. (2002), we experienced a milk protein reduction with GLU infusion. This negative impact on protein and also fat percentages in both EL and LL observed on GLU treatment in our study, could be probably be ascribed to a dilution effect explained by increased lactose synthesis in response to the provision of glucose (Cant et al., 2002; Rook, 1979). There was a significant increase in total milk yield and a numerical increase in ECM in EL but not in LL in response to GLU infusion. This would be in agreement with the mammary gland being capable of increasing glucose uptake to some extent in EL in response to increased supply, but not as efficiently in LL where the synthetic capacity of the mammary gland is presumed to be the most limiting factor for glucose uptake (Nielsen et al., 2001).

Compared to the SAL control group, milk protein yield was increased by provision of EAA and an energy yielding substrate in the form of ACE in EL, but in LL, insufficient mammary EAA supply could apparently not be compensated as efficiently by provision of more energy-yielding substrates. Acetate and glucose are both substrates contributing to oxidative metabolism in the MEC. Acetate and glucose are the main source of energy in the mammary gland of lactating cows and goats, but acetate contributes to the oxidative metabolism more significantly than glucose in both dairy goats and cows (Bickerstaffe et al., 1974). Acetate is utilized in oxidative phosphorylation of adenosine nucleosides, which results in the generation of ATP (Forsberg et al., 1984; Scott et al., 1976), whereas glucose is oxidized mainly through the pentose phosphate pathway to yield NADPH required for de novo fatty acid synthesis (lipogenesis) (Chaiyabutr et al., 1980; Chaiyabutr et al., 2008). Milk protein synthesis is a highly energy consuming process, and 5 ATP are consumed for every peptide bond formed (Hanigan and Baldwin, 1994; Lobley, 1990). Our results confirmed the fourth hypothesis that it is possible in EL to sustain milk protein synthesis whilst substituting amino acid supply to the mammary gland with a substrate, acetate, providing energy predominantly in the form of ATP. But when the energy is given in the form of glucose, protein synthesis and milk protein

content cannot be sustained. This underlines the importance of energy (ATP) supply for milk protein synthesis particularly in EL. The present study thus indicate there could be scope for development of differential protein recommendations for ruminants in early and late lactation, and this issue should be pursued in future studies.

Conclusions and perspectives

Not only MEC renewal, but also development of vascular function, initiation of lactation and growth of the mammary gland continues further into early lactation in the PP compared to the MP dairy goats. This combined with improved MEC survival and maintenance of vascular competence, could explain the reported higher lactation persistency in PP compared to MP goats. We suggest that development and function of the microvasculature may be just as essential for the overall integrity of the mammary gland and lactation persistency as the MEC population. The set of genes regulating these orchestrated changes in MEC and microvascular remodelling, are also basically the ones which can explain the different patterns of mammary development and performance during lactation between PP and MP goats. The factors encoded for by these genes are therefore likely to be important determinants of lactation persistency. The underlying reasons for the differential expression in PP compared to MP goats of genes responsible for regulation of MEC and vascular development remain to be established. Mammary development and remodelling prior to parturition and during lactation in goats are dependent on parity as well as late gestation management (dry period). But, we were not able to resolve why CL during the late gestation period interferes with MEC and vascular remodelling within the mammary gland, and what regulatory mechanisms are responsible. It remains to be elucidated why the full restoration of the MEC population and overall mammary remodelling is inhibited just because of CL, and why this is associated with a marked depression in milk yield in dairy cows in the subsequent lactation, but apparently not in the dairy goat that (with some difficulty) manage to lactate continuously.

In our studies on gene expression we could not identify any specific regulatory factors that would account for the differences in MEC turnover between mammary glands allowed a dry period in late gestation compared to mammary glands milked continuously throughout the late gestation period. However, all gene expression studies so far have been conducted on biopsies of mammary tissue consisting of the variety of different tissues and cell types present in the mammary gland. And this may mask gene expression changes occurring in a particular cell type. To understand the regulation accounting for differences in cell turnover and hence lactation performance in dairy goats subjected to different lactation strategies, it is therefore necessary to study the individual cell types within a given tissue structure in the mammary tissue to be able to obtain a more detailed understanding. For this reason in future studies,

using laser microdissection is recommended to be able to work at the level of individual cell types among different mammary tissue components.

In a tissue structure like an alveoli, it is possible to find apoptotic and some proliferative cells. Laser microdissection makes it possible to go into the individual cells and study their specific characteristics; why was it for example induced to go into apoptosis or proliferation compared to the other cells in that very same tissue structure. This new approach can improve our understanding of what determines the status of a particular cell, where the interactions between different cells could be studied specifically. In this way we could acquire new knowledge about the mechanisms responsible for the regulation of MEC as well as mammary microvasculature remodelling. If we could either synchronize angiogenesis with prepartum MEC proliferation or avoid early lactation apoptosis by knowing those mechanisms, we would have a powerful instrument to improve lactation persistency and overall lactation performance of economical benefit to the producers.

Utilisation of the synthetic capacity present in the mammary gland at any given time point in lactation relies on supply of nutrients by the blood to sustain milk synthesis. We confirmed that the mammary gland synthetic capacity generally appears to be the main limiting factor for milk yield in LL, whereas milk production in EL is more likely to be limited by mammary nutrient supply. Both amino acids and energy in the form of ATP can limit milk protein synthesis and content in EL, and an insufficient supply of amino acids to the mammary gland of dairy goats could in fact be compensated in EL but apparently not LL by increased energy supply to the mammary gland, provided that this increased energy supply was in the form of nutrients like acetate, which are metabolized predominantly in pathways resulting in generation of ATP. In contrast to our initial hypothesis, the mammary gland in EL may thus in fact be more robust towards changes in amino acid supply, capable of maintaining amino acid uptake even at rather low arterial concentrations, whereas higher arterial plasma concentrations of amino acids may be required to sustain an adequate mammary uptake later in lactation. This should be verified in larger scale studies since it is something that potentially could open up for improvements of nitrogen utilization in dairy production through implementation of differential protein recommendations during lactation.

REFERENCES

Akers, R. M. 2000. Selection for milk production from a lactation biology viewpoint. J. Dairy Sci. 83(5):1151-1158.

Akers, R. M. 2002. Lactation and the Mammary Gland. First ed. Blackwell publishing, Ames, Iowa, USA.

Akers, R. M. 2006. Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. J. Dairy Sci. 89(4):1222-1234.

Akers, R. M., A. V. Capuco, and J. E. Keys. 2006. Mammary histology and alveolar cell differentiation during late gestation and early lactation in mammary tissue of beef and dairy heifers. Livestock Science 105(1-3):44-49.

Akers, R. M., and C. W. Heald. 1978. Stimulatory Effect of Prepartum Milk Removal on Secretory Cell-Differentiation in Bovine Mammary-Gland. Journal of Ultrastructure Research 63(3):316-322.

Akers, R. M., and J. E. Keys. 1985. Effect of Suckling Intensity on Human Growth-Hormone Binding, Biochemical-Composition and Histological Characteristics of Ovine Mammary-Glands. Domestic Animal Endocrinology 2(4):159-172.

Allan, G. J., J. Beattie, and D. J. Flint. 2004. The role of IGFBP-5 in mammary gland development and involution. Domestic Animal Endocrinology 27(3):257-266.

Ambrosoli, R., L. Distasio, and P. Mazzocco. 1988. Content of Alpha-S1-Casein and Coagulation Properties in Goat Milk. J. Dairy Sci. 71(1):24-28.

Anderson, R. R., J. R. Harness, A. F. Snead, and M. S. Salah. 1981. Mammary Growth Pattern in Goats during Pregnancy and Lactation. J. Dairy Sci. 64(3):427-432.

Annen, E. L., R. J. Collier, M. A. McGuire, J. L. Vicini, J. M. Ballam, and M. J. Lormore. 2004. Effect of modified dry period lengths and bovine somatotropin on yield and composition of milk from dairy cows. J. Dairy Sci. 87(11):3746-3761.

Annen, E. L., A. C. Fitzgerald, P. C. Gentry, M. A. McGuire, A. V. Capuco, L. H. Baumgard, and R. J. Collier. 2007. Effect of continuous milking and bovine somatotropin supplementation on mammary epithelial cell turnover. J. Dairy Sci. 90(1):165-183.

Annen, E. L., C. M. Stiening, B. A. Crooker, A. C. Fitzgerald, and R. J. Collier. 2008. Effect of continuous milking and prostaglandin E-2 on milk production and mammary epithelial cell turnover, ultrastructure, and gene expression. Journal of Animal Science 86(5):1132-1144.

Bachman, K. C., and M. L. Schairer. 2003. Invited review: Bovine studies on optimal lengths of dry periods. J. Dairy Sci. 86(10):3027-3037.

Baldi, A., S. Modina, F. Cheli, F. Gandolfi, L. Pinotti, L. B. Scesi, F. Fantuz, and V. Dell'Orto. 2002. Bovine somatotropin administration to dairy goats in late lactation:

Effects on mammary gland function, composition and morphology. J. Dairy Sci. 85(5):1093-1102.

Bauman, D. E., and C. L. Davis. 1974. Biosynthesis of milk fat. Pages 31-75 in Lactation, a comprehensive treatise. Vol. 2. B. L. Larsen and V. R. Smith, ed. Academic Press, New York.

Bauman, D. E., I. H. Mather, R. J. Wall, and A. L. Lock. 2006. Major advances associated with the biosynthesis of milk. J. Dairy Sci. 89(4):1235-1243.

Baumrucker, C. R. 2005. Intracrine signaling in the mammary gland. Livestock Production Science 98(1-2):47-56.

Baumrucker, C. R., and N. E. Erondu. 2000. Insulin-like growth factor (IGF) system in the bovine mammary gland and milk. Journal of Mammary Gland Biology and Neoplasia 5(1):53-64.

Baumrucker, C. R., C. A. Gibson, and F. L. Schanbacher. 2003. Bovine lactoferrin binds to insulin-like growth factor-binding protein-3. Domestic Animal Endocrinology 24(4):287-303.

Bequette, B. J., F. R. C. Backwell, and L. A. Crompton. 1998. Current concepts of amino acid and protein metabolism in the mammary gland of the lactating ruminant. J. Dairy Sci. 81(9):2540-2559.

Bequette, B. J., J. A. Metcalf, D. WrayCahen, F. R. C. Backwell, J. D. Sutton, M. A. Lomax, J. C. Macrae, and G. E. Lobley. 1996. Leucine and protein metabolism in the lactating dairy cow mammary gland: Responses to supplemental dietary crude protein intake. Journal of Dairy Research 63(2):209-222.

Bickerstaffe, R., E. F. Annison, and J. L. Linzell. 1974. Metabolism of Glucose, Acetate, Lipids and Amino-Acids in Lactating Dairy-Cows. Journal of Agricultural Science 82(FEB):71-85.

Bortner, C. D., N. B. E. Oldenburg, and J. A. Cidlowski. 1995. The Role of Dna Fragmentation in Apoptosis. Trends in Cell Biology 5(1):21-26.

Boutinaud, M., J. Guinard-Flament, and H. Jammes. 2004. The number and activity of mammary epithelial cells, determining factors for milk production. Reproduction Nutrition Development 44(5):499-508.

Bursch, W., S. Paffe, B. Putz, G. Barthel, and R. Schultehermann. 1990. Determination of the Length of the Histological Stages of Apoptosis in Normal Liver and in Altered Hepatic Foci of Rats. Carcinogenesis 11(5):847-853.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25(2):169-193.

Butt, A. J., and A. C. Williams. 2001. IGFBP-3 and apoptosis - a licence to kill ? Apoptosis 6(3):199-205.

Caja, G., A. A. K. Salama, and X. Such. 2006. Omitting the dry-off period negatively affects colostrum and milk yield in dairy goats. J. Dairy Sci. 89(11):4220-4228.

Cant, J. P., D. R. Trout, F. Qiao, and N. G. Purdie. 2002. Milk synthetic response of the bovine mammary gland to an increase in the local concentration of arterial glucose. J. Dairy Sci. 85(3):494-503.

Cant, J. R., R. Berthiaume, H. Lapierre, P. H. Luimes, B. W. McBride, and D. Pacheco. 2003. Responses of the bovine mammary glands to absorptive supply of single amino acids. Canadian Journal of Animal Science 83(3):341-355.

Capuco, A. V., and R. M. Akers. 1990. Thymidine Incorporation by Lactating Mammary Epithelium During Compensatory Mammary Growth in Beef-Cattle. J. Dairy Sci. 73(11):3094-3103.

Capuco, A. V., and R. M. Akers. 1999. Mammary involution in dairy animals. Journal of Mammary Gland Biology and Neoplasia 4(2):137-144.

Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in Holstein cows during the dry period: Quantification of nucleic acids and histology. J. Dairy Sci. 80(3):477-487.

Capuco, A. V., S. E. Ellis, S. A. Hale, E. Long, R. A. Erdman, X. Zhao, and M. J. Paape. 2003. Lactation persistency: Insights from mammary cell proliferation studies. Journal of Animal Science 81:18-31.

Capuco, A. V., D. L. Wood, R. Baldwin, K. Mcleod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bST. J. Dairy Sci. 84(10):2177-2187.

Cassy, S., M. Charlier, L. Belair, M. Guillomot, K. Laud, and J. Djiane. 2000. Increase in prolactin receptor (PRL-R) mRNA level in the mammary gland after hormonal induction of lactation in virgin ewes. Domestic Animal Endocrinology 18(1):41-55.

Chaiyabutr, N., A. Faulkner, and M. Peaker. 1980. Utilization of Glucose for the Synthesis of Milk Components in the Fed and Starved Lactating Goat Invivo. Biochemical Journal 186(1):301-308.

Chaiyabutr, N., S. Komolvanich, S. Thammacharoen, and S. Chanpongsang. 2008. Effects of long-term exogenous bovine somatotropin on glucose metabolism and the utilization of glucose by the mammary gland in different stages of lactation of crossbred Holstein cattle. Animal Science Journal 79(5):561-574.

Chilliard, Y., M. Bonnet, C. Delavaud, Y. Faulconnier, C. Leroux, J. Djiane, and F. Bocquier. 2001. Leptin in ruminants. Gene expression in adipose tissue and mammary gland, and regulation of plasma concentration. Domestic Animal Endocrinology 21(4):271-295.

Chilliard, Y., C. Delouis, M. C. Smith, D. Sauvant, and P. Morandfehr. 1986. Mammary Metabolism in the Goat During Normal Or Hormonally-Induced Lactation. Reproduction Nutrition Development 26(2B):607-615.

Chilliard, Y., A. Ferlay, J. Rouel, and G. Lamberett. 2003. A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. J. Dairy Sci. 86(5):1751-1770.

Claria, J. 2003. Cyclooxygenase-2 biology. Current Pharmaceutical Design 9(27):2177-2190.

Conway, E. M., D. Collen, and P. Carmeliet. 2001. Molecular mechanisms of blood vessel growth. Cardiovasc Res 49(3):507-521.

Cvek, K., K. Dahlborn, and Y. Ridderstrale. 1998. Localization of carbonic anhydrase in the goat mammary gland during involution and lactogenesis. Journal of Dairy Research 65(1):43-54.

Danfaer, A., V. Tetens, and N. Agergaard. 1995. Review and An Experimental-Study on the Physiological and Quantitative Aspects of Gluconeogenesis in Lactating Ruminants. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 111(2):201-210.

Djonov, V., A. C. Andres, and A. Ziemiecki. 2001. Vascular remodelling during the normal and malignant life cycle of the mammary gland. Microscopy Research and Technique 52(2):182-189.

Ellis, S., and A. V. Capuco. 2002. Cell proliferation in bovine mammary epithelium: identification of the primary proliferative cell population. Tissue & Cell 34(3):155-163.

ELsayed, E. H., M. H. EL-Shafie, E. O. H. Saifelnasr, and A. A. bu El-Ella. 2009. Histological and histochemical study on mammary gland of Damascus goats through stages of lactation. Small Ruminant Research 85(1):11-17.

Erb, R. E. 1977. Hormonal-Control of Mammogenesis and Onset of Lactation in Cows - Review. J. Dairy Sci. 60(2):155-169.

Fitzgerald, A. C., E. L. nnen-Dawson, L. H. Baumgard, and R. J. Collier. 2007. Evaluation of continuous lactation and increased milking frequency on milk production and mammary cell turnover in primiparous Holstein cows. J. Dairy Sci. 90(12):5483-5489.

Fleet, I. R., and T. B. Mepham. 1983. Physiology Methods Used in the Study of Mammary Substrate Utilization in Ruminants. Pages 469-491 in Biochemistry of Lactation. T. B. Mepham, ed. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Forsberg, N. E., R. L. Baldwin, and N. E. Smith. 1984. Roles of Acetate and Its Interactions with Glucose and Lactate in Cow Mammary Tissue. J. Dairy Sci. 67(10):2247-2254.

Forsyth, I. A., and M. C. Neville. 2009. Introduction: Hormonal Regulation of Mammary Development and Milk Protein Gene Expression at the Whole Animal and Molecular Levels. Journal of Mammary Gland Biology and Neoplasia 14(3):317-319.

Fowler, P. A., C. H. Knight, and M. A. Foster. 1991. Omitting the Dry Period Between Lactations Does Not Reduce Subsequent Milk-Production in Goats. Journal of Dairy Research 58(1):13-19.

Freeman, T. C., K. Lee, and P. J. Richardson. 1999. Analysis of gene expression in single cells. Current Opinion in Biotechnology 10(6):579-582.

Gerdes, J., L. Li, C. Schlueter, M. Duchrow, C. Wohlenberg, C. Gerlach, I. Stahmer, S. Kloth, E. Brandt, and H. D. Flad. 1991. Immunobiochemical and Molecular Biologic Characterization of the Cell Proliferation-Associated Nuclear Antigen That Is Defined by Monoclonal-Antibody Ki-67. American Journal of Pathology 138(4):867-873.

Grill, C. J., and W. S. Cohick. 2000. Insulin-like growth factor binding protein-3 mediates IGF-I action in a bovine mammary epithelial cell line independent of an IGF interaction. Journal of Cellular Physiology 183(2):273-283.

Guinard, J., and H. Rulquin. 1994a. Effect of Graded-Levels of Duodenal Infusions of Casein on Mammary Uptake in Lactating Cows .2. Individual Amino-Acids. J. Dairy Sci. 77(11):3304-3315.

Guinard, J., and H. Rulquin. 1994b. Effects of Graded Amounts of Duodenal Infusions of Lysine on the Mammary Uptake of Major Milk Precursors in Dairy-Cows. J. Dairy Sci. 77(12):3565-3576.

Gundersen, H. J. G., T. F. Bendtsen, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J. R. Nyengaard, B. Pakkenberg, F. B. Sorensen, A. Vesterby, and M. J. West. 1988. Some New, Simple and Efficient Stereological Methods and Their Use in Pathological Research and Diagnosis - Review Article. Apmis 96(5):379-394.

Hamann, J., and J. Reichmuth. 1990. Compensatory Milk-Production Within the Bovine Udder - Effects of Short-Term Nonmilking of Single Quarters. Journal of Dairy Research 57(1):17-22.

Hanigan, M. D., and R. L. Baldwin. 1994. A Mechanistic Model of Mammary-Gland Metabolism in the Lactating Cow. Agricultural Systems 45(4):369-419.

Hanigan, M. D., B. J. Bequette, L. A. Crompton, and J. France. 2001. Modeling mammary amino acid metabolism. Livestock Production Science 70(1-2):63-78.

Harness, J. R., and R. R. Anderson. 1977. Effects of Relaxin in Combination with Prolactin and Ovarian Steroids on Mammary Growth in Hypophysectomized Rats. Proceedings of the Society for Experimental Biology and Medicine 156(2):354-358.

Henderson, A. J., and M. Peaker. 1983. Compensatory Increases in Milk Secretion in Response to Unilateral Inhibition by Colchicine During Lactation in the Goat. Journal of Physiology-London 334(JAN):433-440.

Howard, C. V., and M. G. Reed. 2005. Unbiased Stereology. Second ed. Garland Science/BIOS Scientific Publishers, Cornwall, UK.

Hurley, W. L. 1989. Mammary-Gland Function During Involution. J. Dairy Sci. 72(6):1637-1646.

Janqueira, L. C., J. Carneiro, and R. O. Kelly. 1995. Basic Histology. 8th ed. Prentice-Hall International, New Jersy.

Jenness, R. 1974. Biosynthesis and Composition of Milk. Journal of Investigative Dermatology 63(1):109-118.

Keys, J. E., A. V. Capuco, R. M. Akers, and J. Djiane. 1989. Comparative-Study of Mammary-Gland Development and Differentiation Between Beef and Dairy Heifers. Domestic Animal Endocrinology 6(4):311-319.

King, R. W., R. J. Deshaies, J. M. Peters, and M. W. Kirschner. 1996. How proteolysis drives the cell cycle. Science 274(5293):1652-1659.

Knight, C. H. 2000. The importance of cell division in udder development and lactation. Livestock Production Science 66(2):169-176.

Knight, C. H., J. France, and D. E. Beever. 1994. Nutrient Metabolism and Utilization in the Mammary-Gland. Livestock Production Science 39(1):129-137.

Knight, C. H., and M. Peaker. 1984. Mammary Development and Regression During Lactation in Goats in Relation to Milk Secretion. Quarterly Journal of Experimental Physiology and Cognate Medical Sciences 69(2):331-338.

Knight, C. H., and C. J. Wilde. 1993. Mammary Cell Changes During Pregnancy and Lactation. Livestock Production Science 35(1-2):3-19.

Kuhn, N. J. 1983. The Biosynthessis of Lactose. Pages 159-176 in Biochemistry of Lactation. T. B. Mepham, ed. Elsevier Sience Publishers B. V., Amsterdam, The Netherlands.

Law, A. J. R., and J. R. Brown. 1994. Compositional Changes in Caprine Whey Proteins. Milchwissenschaft-Milk Science International 49(12):674-678.

Lawrence, T. L. J., and V. R. Fowler. 2002. Growth of Farm Animals. 2nd ed. CABI Publishing, New York.

Linzell, J. L. 1960. Valvular Incompetence in the Venous Drainage of the Udder. Journal of Physiology-London 153(3):481-&.

Littlefield, J. W., and E. A. Gould. 1960. Toxic Effect of 5-Bromodeoxyuridine on Cultured Epithelial Cells. Journal of Biological Chemistry 235(4):1129-1133.

Lobley, G. E. 1990. Energy-Metabolism Reactions in Ruminant Muscle - Responses to Age, Nutrition and Hormonal Status. Reproduction Nutrition Development 30(1):13-34.

Lock, A. L., and D. E. Bauman. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids 39(12):1197-1206.

Lockey, C., E. Otto, and Z. F. Long. 1998. Real-time fluorescence detection of a single DNA molecule. Biotechniques 24(5):744-746.

Lun. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill Book Inc., New York.

Mackenzie, D. 1967. Goat Husbandry. 2nd ed. Faber & Faber, London.

Madsen, T. G. 2002. Regulation of mammary nutrient uptake and metabolism in ruminants. PhD , The Royal Veterinary and Agricultural University.

Madsen, T. G., L. Nielsen, and M. O. Nielsen. 2005. Mammary nutrient uptake in response to dietary supplementation of rumen protected lysine and methionine in late and early lactating dairy goats. Small Ruminant Research 56(1-3):151-164.

Madsen, T. G., M. O. Nielsen, J. B. Andersen, and K. L. Ingvartsen. 2008. Continuous lactation in dairy cows: Effect on milk production and mammary nutrient supply and extraction. J. Dairy Sci. 91(5):1791-1801.

Maher, S., D. Toomey, C. Condron, and D. Bouchier-Hayes. 2002. Activation-induced cell death: The controversial role of Fas and Fas ligand in immune privilege and tumour counterattack. Immunology and Cell Biology 80(2):131-137.

Matsumoto, M., H. Nishinakagawa, M. Kurohmaru, Y. Hayashi, and J. Otsuka. 1992. Pregnancy and Lactation Affect the Microvasculature of the Mammary-Gland in Mice. Journal of Veterinary Medical Science 54(5):937-943.

McFadden, T. B., R. M. Akers, and G. W. Kazmer. 1987. Alpha-Lactalbumin in Bovine Serum - Relationships with Udder Development and Function. J. Dairy Sci. 70(2):259-264.

McManaman, J. L., and M. C. Neville. 2003. Mammary physiology and milk secretion. Advanced Drug Delivery Reviews 55(5):629-641.

Mepham, T. B. 1982. Amino-Acid Utilization by Lactating Mammary-Gland. J. Dairy Sci. 65(2):287-298.

Miller, N., L. Delbecchi, D. Petitclerc, G. F. Wagner, B. G. Talbot, and P. Lacasse. 2006. Effect of stage of lactation and parity on mammary gland cell renewal. J. Dairy Sci. 89(12):4669-4677.

Miller, P. S., B. L. Reis, C. C. Calvert, E. J. Depeters, and R. L. Baldwin. 1991. Patterns of Nutrient-Uptake by the Mammary-Glands of Lactating Dairy-Cows. J. Dairy Sci. 74(11):3791-3799.

Mioc, B., Z. Prpic, I. Vnucec, Z. Barac, V. Susic, D. Samarzija, and V. Pavic. 2008. Factors affecting goat milk yield and composition. Mljekarstvo 58(4):305-313.

Neville, M. C., T. B. McFadden, and I. Forsyth. 2002. Hormonal regulation of mammary differentiation and milk secretion. Journal of Mammary Gland Biology and Neoplasia 7(1):49-66.

Neville, M. C., and M. F. Picciano. 1997. Regulation of milk lipid secretion and composition. Annual Review of Nutrition 17:159-183.

Nielsen, M. O., I. R. Fleet, K. Jakobsen, and R. B. Heap. 1995a. The Local Differential Effect of Prostacyclin, Prostaglandin E(2) and Prostaglandin-F2-Alpha on Mammary Blood-Flow of Lactating Goats. Journal of Endocrinology 145(3):585-591.

Nielsen, M. O., and K. Jakobsen. 1993. Changes in Mammary Glucose and Protein-Uptake in Relation to Milk Synthesis During Lactation in High-Yielding and Low-Yielding Goats. Comparative Biochemistry and Physiology A-Physiology 106(2):359-365.

Nielsen, M. O., K. Jakobsen, and J. N. Jorgensen. 1990. Changes in Mammary Blood-Flow During the Lactation Period in Goats Measured by the Ultrasound Doppler Principle. Comparative Biochemistry and Physiology A-Physiology 97(4):519-524.

Nielsen, M. O., T. G. Madsen, and A. M. Hedeboe. 2001. Regulation of mammary glucose uptake in goats: role of mammary gland supply, insulin, IGF-1 and synthetic capacity. Journal of Dairy Research 68(3):337-349.

Nielsen, M. O., S. Nyborg, K. Jakobsen, I. R. Fleet, and J. Norgaard. 2004a. Mammary uptake and excretion of prostanoids in relation to mammary blood flow and milk yield during pregnancy-lactation and somatotropin treatment in dairy goats. Domestic Animal Endocrinology 27(4):345-362.

Nielsen, M. O., S. Nyborg, K. Jakobsen, I. R. Fleet, and J. Norgaard. 2004b. Mammary uptake and excretion of prostanoids in relation to mammary blood flow and milk yield during pregnancy-lactation and somatotropin treatment in dairy goats. Domestic Animal Endocrinology 27(4):345-362.

Nielsen, M. O., C. Schleisner, K. Jakobsen, and P. H. Andersen. 1995b. The effect of mammary O-2 uptake, CO2 and H+ production on mammary blood flow during pregnancy, lactation and somatotropin treatment in goats. Comparative Biochemistry and Physiology A-Physiology 112(3-4):591-599.

Nishinak, H. 1970. Blood Supply to Mammary Glands of Mouse .3. Effects of Ovariectomy. Japanese Journal of Veterinary Science 32:80-&.

Norgaard, J., A. Sorensen, M. T. Sorensen, J. B. Andersen, and K. Sejrsen. 2005. Mammary cell turnover and enzyme activity in dairy cows: Effects of milking frequency and diet energy density. J. Dairy Sci. 88(3):975-982.

Norgaard, J. V. 2007. Factors affecting mammary cell turnover and cell activity during the pregnancy-lactation cycle in ruminants. PhD , University of Copenhagen.

Norgaard, J. V., M. O. Nielsen, P. K. Theil, M. T. Sorensen, S. Safayi, and K. Sejrsen. 2008a. Development of mammary glands of fat sheep submitted to restricted feeding during late pregnancy. Small Ruminant Research 76(3):155-165.

Norgaard, J. V., P. K. Theil, M. T. Sorensen, and K. Sejrsen. 2008b. Cellular mechanisms in regulating mammary cell turnover during lactation and dry period in dairy cows. J. Dairy Sci. 91(6):2319-2327.

Patton, S. 1969. Milk. Scientific American 221(1):59-&.

Pepper, M. S., D. Baetens, S. J. Mandriota, C. Di Sanza, S. Oikemus, T. F. Lane, J. V. Soriano, and R. Montesano. 2000b. Regulation of VEGF and VEGF receptor expression in the rodent mammary gland during pregnancy, lactation, and involution. Developmental Dynamics 218(3):507-524.

Pepper, M. S., D. Baetens, S. J. Mandriota, C. Di Sanza, S. Oikemus, T. F. Lane, J. V. Soriano, and R. Montesano. 2000a. Regulation of VEGF and VEGF receptor expression in the rodent mammary gland during pregnancy, lactation, and involution. Developmental Dynamics 218(3):507-524.

Plath, A., R. Einspanier, F. Peters, F. Sinowatz, and D. Schams. 1997. Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation. Journal of Endocrinology 155(3):501-511.

Proskuryakov, S. Y., A. G. Konoplyannikov, and V. L. Gabai. 2003. Necrosis: a specific form of programmed cell death? Experimental Cell Research 283(1):1-16.

Radcliff, R. P., M. J. VandeHaar, L. T. Chapin, T. E. Pilbeam, D. K. Beede, E. P. Stanisiewski, and H. A. Tucker. 2000. Effects of diet and injection of bovine somatotropin on prepubertal growth and first-lactation milk yields of Holstein cows. J. Dairy Sci. 83(1):23-29.

Rasmussen, A. N., M. O. Nielsen, A. H. Tauson, H. Offenberg, P. D. Thomsen, and D. Blache. 2008. Mammary gland leptin in relation to lactogenesis in the periparturient dairy goat. Small Ruminant Research 75(1):71-79.

Reed, J. C. 1998. Bcl-2 family proteins. Oncogene 17(25):3225-3236.

Remond, B., and J. C. Bonnefoy. 1997. Performance of a herd of Holstein cows managed without the dry period. Annales de Zootechnie 46(1):3-12.

Remond, B., J. Kerouanton, and V. Brocard. 1997. The effect of reducing or omitting the dry period on the performance of dairy cows. Productions Animales 10(4):301-315.

Riley, L. G., P. C. Wynn, P. Williamson, and P. A. Sheehy. 2008. The role of native bovine alpha-lactalbumin in bovine mammary epithelial cell apoptosis and casein expression. Journal of Dairy Research 75(3):319-325.

Rook, J. A. F. 1979. Role of Carbohydrate-Metabolism in the Regulation of Milk-Production. Proceedings of the Nutrition Society 38(3):309-314.

Rudolph, M. C., J. L. McManaman, T. Phang, T. Russell, D. J. Kominsky, N. J. Serkova, T. Stein, S. M. Anderson, and M. C. Neville. 2007. Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. Physiological Genomics 28(3):323-336.

Rulquin, H., P. M. Pisulewski, R. Verite, and J. Guinard. 1993. Milk-Production and Composition As A Function of Postruminal Lysine and Methionine Supply - A Nutrient-Response Approach. Livestock Production Science 37(1-2):69-90.

Sauvant, D., and P. Morand-Fehr. 1989. Goats. Pages 169-180 in Ruminant Nutrition: Recommended Allowances and Feed Tables. R. Jarrige, ed. John Libbey and Co., Ltd., London.

Schmittgen, T. D., B. A. Zakrajsek, A. G. Mills, V. Gorn, M. J. Singer, and M. W. Reed. 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods. Analytical Biochemistry 285(2):194-204.

Schultz, D. R., and W. J. Harrington. 2003. Apoptosis: Programmed cell death at a molecular level. Seminars in Arthritis and Rheumatism 32(6):345-369.

Scott, R. A., D. E. Bauman, and J. H. Clark. 1976. Cellular Gluconeogenesis by Lactating Bovine Mammary Tissue. J. Dairy Sci. 59(1):50-56.

Sejrsen, K. 1994. Relationships Between Nutrition, Puberty and Mammary Development in Cattle. Proceedings of the Nutrition Society 53(1):103-111.

Sell, C., R. Baserga, and R. Rubin. 1995. Insulin-Like Growth-Factor-I (Igf-I) and the Igf-I Receptor Prevent Etoposide-Induced Apoptosis. Cancer Research 55(2):303-306.

Seymour, W. M., C. E. Polan, and J. H. Herbein. 1990. Effects of Dietary-Protein Degradability and Casein Or Amino-Acid Infusions on Production and Plasma Amino-Acids in Dairy-Cows. J. Dairy Sci. 73(3):735-748.

Sharma, R., and V. K. Kansal. 2000. Heterogeneity of cationic amino acid transport systems in mouse mammary gland and their regulation by lactogenic hormones. Journal of Dairy Research 67(1):21-30.

Sheffield, L. G. 1988. Organization and Growth of Mammary Epithelia in the Mammary-Gland Fat Pad. J. Dairy Sci. 71(10):2855-2874.

Shennan, D. B., and M. Peaker. 2000. Transport of milk constituents by the mammary gland. Physiological Reviews 80(3):925-951.

Sherr, C. J. 1995. D-Type Cyclins. Trends in Biochemical Sciences 20(5):187-190.

Shibuya, M. 2001. Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. Cell Structure and Function 26(1):25-35.

Silva, L. F. P., M. J. VandeHaar, M. S. W. Nielsen, and G. W. Smith. 2002. Evidence for a local effect of leptin in bovine mammary gland. J. Dairy Sci. 85(12):3277-3286.

Simmons, D. L., R. M. Botting, and T. Hla. 2004. Cyclooxygenase isozymes: The biology of prostaglandin synthesis and inhibition. Pharmacological Reviews 56(3):387-437.

Sinha, Y. N., and H. A. Tucker. 1969. Mammary Development and Pituitary Prolactin Level of Heifers from Birth Through Puberty and During Estrous Cycle. J. Dairy Sci. 52(4):507-&.

Smith, J. A., and L. Martin. 1973. Do Cells Cycle. Proceedings of the National Academy of Sciences of the United States of America 70(4):1263-1267.

Smith, K. L., and D. A. Todhunter. 1985. The physiology of mammary gland during the dry period and the relationship to infection. Pages 87 in Vol. Louisville, KY, USA Natl. Mastitis Counc., Arlington, VA, USA.

Soemarwoto, I. N., and H. A. Bern. 1958. The Effect of Hormones on the Vascular Pattern of the Mouse Mammary Gland. American Journal of Anatomy 103(3):403-435.

Sorensen, M. T., J. V. Norgaard, P. K. Theil, M. Vestergaard, and K. Sejrsen. 2006. Cell turnover and activity in mammary tissue during lactation and the dry period in dairy cows. J. Dairy Sci. 89(12):4632-4639.

Strange, R., F. Li, S. Saurer, A. Burkhardt, and R. R. Friis. 1992. Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development 115(1):49-58.

Strudsholm, F., O. Aaes, J. Madsen, V. F. Kristensen, H. R. Andersen, T. Hvelplund, and S. Oestergaard. 1999. Danske fodernormer till Kvaeg (Danish feed recommendations for cattle). Pages 1-47 in Report No. 84. Landsudvalget for Kvaeg, Aarhus.

Swanson, E. W. 1965. Comparing Continuous Milking with 60-Day Dry Periods in Successive Lactations. J. Dairy Sci. 48(9):1205-&.

Tevfik Dorak, M. 2007. Real-time PCR. 1st ed. Taylor & Francis Group.

Theil, P. K., K. Sejrsen, W. L. Hurley, R. Labouriau, B. Thomsen, and M. T. Sorensen. 2006. Role of suckling in regulating cell turnover and onset and maintenance of lactation in individual mammary glands of sows. Journal of Animal Science 84(7):1691-1698.

Thurston, G. 2003. Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. Cell and Tissue Research 314(1):61-68.

Tucker, H. A. 1981. Physiological Control of Mammary Growth, Lactogenesis, and Lactation. J. Dairy Sci. 64(6):1403-1421.

Tucker, H. A. 1987. Quantitative Estimates of Mammary Growth During Various Physiological States: A Review. J. Dairy Sci. 70(9):1958-1966.

Uden, P., and A. Danfaer. 2008. Modeling glucose metabolism in the dairy cow - A comparison of two dynamic models. Animal Feed Science and Technology 143(1-4):59-69.

Van Cruchten, S., and W. Van den Broeck. 2002. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. Anatomia Histologia Embryologia-Journal of Veterinary Medicine Series C 31(4):214-223.

Vermeulen, K., D. R. Van Bockstaele, and Z. N. Berneman. 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Proliferation 36(3):131-149.

Wall, E. H., H. M. Crawford, S. E. Ellis, G. E. Dahl, and T. B. McFadden. 2006. Mammary response to exogenous prolactin or frequent milking during early lactation in dairy cows. J. Dairy Sci. 89(12):4640-4648.

Ward, P. P., E. Paz, and O. M. Conneely. 2005. Multifunctional roles of lactoferrin: a critical overview. Cellular and Molecular Life Sciences 62(22):2540-2548.

Wareski, P., T. Motyl, Z. Ryniewicz, A. Orzechowski, B. Gajkowska, U. Wojewodzka, and T. Ploszaj. 2001. Expression of apoptosis-related proteins in mammary gland of goat. Small Ruminant Research 40(3):279-289.

Waterhouse, N. J., J. E. Ricci, and D. R. Green. 2002. And all of a sudden it's over: mitochondrial outer-membrane permeabilization in apoptosis. Biochimie 84(2-3):113-121.

Wathes, D. C., Z. Cheng, N. Bourne, V. J. Taylor, M. P. Coffey, and S. Brotherstone. 2007. Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. Domestic Animal Endocrinology 33(2):203-225.

Weekes, T. L., P. H. Luimes, and J. P. Cant. 2006. Responses to amino acid imbalances and deficiencies in lactating dairy cows. J. Dairy Sci. 89(6):2177-2187.

Wilde, C. J., A. J. Henderson, and C. H. Knight. 1986. Metabolic Adaptations in Goat Mammary Tissue During Pregnancy and Lactation. Journal of Reproduction and Fertility 76(1):289-298.

Wilde, C. J., and C. H. Knight. 1989. Metabolic Adaptations in Mammary-Gland During the Declining Phase of Lactation. J. Dairy Sci. 72(6):1679-1692.

Williams, G. T., C. A. Smith, E. Spooncer, T. M. Dexter, and D. R. Taylor. 1990. Hematopoietic Colony Stimulating Factors Promote Cell-Survival by Suppressing Apoptosis. Nature 343(6253):76-79.

Yasugi, T., T. Kaido, and Y. Uehara. 1989. Changes in Density and Architecture of Microvessels of the Rat Mammary-Gland During Pregnancy and Lactation. Archives of Histology and Cytology 52(2):115-122.

Appendixes

Appendix 1

Biopsy sampling

Before taking biopsies using a fine needle biopsy gun (BIOPTY, Radiplast AB, Uppsala, Sweden, 14G x 10 cm biopsy needle, length of sample notch approximately 1.9 cm), we washed the target area by a scrub-brush containing anti-microbial skin cleaner, shaving, disinfecting with iodine, marking the insertion point, making local anaesthesia (0.5 ml Lidocain 20 mg/ml for each side), and penetrating skin by using a large gauge needle (18G). To avoid insertion of biopsy needle in major blood vessels (basement and midline of the udder) and ductal area (nipples dorsal part), we were pulling the nipples in ventral and then cranial direction, and pushing it towards the sampler (caudally) by pressing the cranial part. After sampling, if there was no bleeding, we were using Penicillin powder and then Wound Plast to make a cover on the wound. The animal was milked, both before (to avoid milk in the biopsy) and after sampling (to remove any blood clots in the udder).

Note:

- It was tried to take samples from the same site of the gland at each sampling, by recording its position compared to the base- and midline of the udder. Of course, the size of the gland varies at different stages of gestation-lactation and doing so, was most helpful when the times of samplings were for stages close to each other, especially around the parturition time.

Appendix 2

Tissue preparation

The mammary tissue samples were fixed in 4% para-formaldehyde (PFA) solution (see appendix 2) for one day, then dehydrated in graded series of ethanol (70%, 80%, 95% and 100%) (2 × 60 min each), cleared in xylene (3 × 60 min), infiltrated with paraffin wax (56-58 ºC) (2 × 90 min), and embedded in paraffin (Histokinet, Shandon Citadel 2000, UK). Samples were further sectioned in sections of 4 μm thickness (Microtome, Jung, Germany). Slides were dried in 37°C oven over two nights before being stained or stored.

Appendix 3

Preparation of 4% PFA

- Solution 1
NaH_2PO_4 5,52 g (NaH2PO4, H2O = 6,35g)
Milli-Q water 200 ml

- Solution 2
Na_2HPO_4 28,83 g
Milli-Q water 810 ml

- Prepare 2L 0.1M PO_4-buffer pH7,4
Adjust Solution 2 to pH 7.4 with solution 1
Dilute solution 2 (pH=7.4) with water 1:2 (1 liter Solution 2 (pH=7,4) + 1 liter Milli-Q water)

- Prepare 4% paraformaldehyd in 0.1M PO_4-buffer pH 7,4. (=4%PFA)
Paraformaldehyd 20 g
0.1M PO_4-buffer pH 7.4 500 ml
Place it on a heater with magnet (3-4 hours) until the solution is clear.
Before use, place the 4%PFA in the refrigerator.

Appendix 4

Mayer's Haematoxylin-Eosin (H&E) method
(Procedure for goat mammary samples)
This is a combination of hematoxylin and eosin (H&E). Hematoxylin stains the acidic structures of the cell, including the (chromatin within) nucleus and the parts of cytoplasm which are rich in RNA, in a blue colour (Janqueira et al., 1995). In contrast, eosin stains the cytoplasm in red and collagen in pink. H&E is one of the most commonly used methods in histology (Lun., 1968), giving details of various components in the tissue. In our studies (papers 1 and 3), various components such as alveoli and its lumen in general and MEC and its grade of differentiation in particular have been quantified or qualified using H&E slides. But, H&E stains glycogens weakly, if at all. That's why another staining method, PAS has been tried (explained below), to be able to distinguish micro-vessels more clearly in the slides.

Step	Procedure	Time *(min.)*
1	Xylene I	5
2	Xylene II	5
3	Xylene III	5
4	Absolute alcohol I	5
5	Absolute alcohol II	5
6	Absolute alcohol III	5
7	96% Alcohol	2
8*	70% Alcohol	5
9	Distilled water	5
10	Staining in haematoxylin	30
11	Washing with running tap water	5
12	Differentiation in Acid-Alcohol	2-4 times (dip)
13	Washing with running tap water	5-10
14	Distilled water	2
15*	Dehydration in 70% alcohol	2
16	Counterstaining in eosin 1%	5
17	Dehydration in 96% alcohol	5 times (wash)
18	Dehydration in absolute alcohol I	2
19	Dehydration in absolute alcohol II	5
20	Dehydration in absolute alcohol III	5
21	Cleared in xylene I	5
22	Cleared in xylene II	5
23	Cleared in xylene III	5-…
24	*Mounting with xylene based medium (Covering)*	

Notes:

- Steps 1-3, 4-6, 18-20 and 21-23, each in 3 separate jars.
- Steps *8,*15- The same work.
- Step 10- Mayers, BIE&B: LAB 12735. – Filtering every day, Use only 5 times!
- Step 13- The running tap water should flow in a moderate stream, neither too much nor too little.
- Step 16- Filtering every day!

- Step 24- BIE&BERNSTEN A-S, DPX Mountant, BDH, Prod 360294H – 500 ml.
- Step 24- The mounting at the end should be done in the fume cupboard, taking care the mounting solution does not enter the rubber tip as this dissolves over time as it contains xylene (i.e do not press the tip too hard when the pipette is in the bottle).
- Step 24- Make sure there are no air bubbles between the mounting glass and the tissue, eventual bubbles can be ‘squeezed’ out pressing on the mounting glass.
- Step 24- Leave the mounted slides 2 days in the fume cupboard before use.

Appendix 5

Periodic Acid-Schiff Reaction (PAS)
(Procedure for goat mammary samples)

Periodic acid-schiff (PAS) reaction demonstrates glycogen, a ubiquitous polysaccharide in the body, not bound to a protein or lipid. The PAS reaction, based on the oxidative action of periodic acid (HIO_4) on 1, 2-glycol groups present in the glucose residues (carbohydrates), gives rise to aldehyde groups that react with Schiffs reagent, providing a new complex compound with a purple or magenta colour (Janqueira et al., 1995). That is why PAS is a staining method preliminary used to identify the tissue structures like vessel walls to which the carbohydrates are attached.

Step	Procedure[#]	Time *(min.)*
1	Xylene I	5
2	Xylene II	5
3	Xylene III	5
4	Absolute Alcohol I	5
5	Absolute Alcohol II	5
6	Absolute Alcohol III	5
7	96% Alcohol	2
*8	70% Alcohol	5
9	Distilled Water	5
10	Oxidization in periodic acid 0.5%	5
11	Washing in running tap water	5
12	Rinsing in distilled water	Once
13	Schiff Solution	20
14	Washing in running tap water	5
15	Rinsing in distilled water	Once
16	Counterstaining Mayer's haematoxylin	3
17	Washing in running tap water	5
18	Differentiation in 1% Acid-Alcohol	3 (Dip)
19	Washing in running tap water	10
20	Rinsing in distilled water	Once
*21	Dehydration in 70% alcohol	2
22	Dehydration in 96% alcohol	2
23	Dehydration in absolute alcohol I	2
24	Dehydration in absolute alcohol II	5
25	Dehydration in absolute alcohol III	5
26	Cleared in xylene I	5
27	Cleared in xylene II	5
28	Cleared in xylene III	5-…
29	*Mounting with xylene based medium (Covering)*	…

Notes:

- 1-3, 4-6, 23-25 and 26-28, each in 3 separate jars
- *8,*21- The same work.

- 10- Merck: 100524 – 25 g – $H_5 10_6$, 0,75 g + 150 ml H_2O every day.
- 13- Merck: 109033 – 500 ml.
- 16- Mayers, BIE&B: LAB 12735.
- 18- HCl - 96% or 70% Ethanol (880 ml 70% Eth. + 24 ml HCl (concentrated)).
- 29- BIE&BERNSTEN A-S, DPX Mountant, BDH, Prod 360294H – 500 ml.

Appendix 6

Immunohistochemistry methods Ki-67 and TUNEL

Ki-67. All cells are able to express Ki-67 protein during different phases of the cell cycle except in phase G_0 (Gerdes et al., 1991). Therefore, labelling of this protein is considered a good marker of cell proliferation. 5-bromodeoxyuridine (BrdU) is another way of labelling the proliferation in the cell, which binds to the single strand DNA in S phase, and therefore, gives a narrower result compare to Ki-67 which could be localized from G_1, S, G_2 and M phases. But, the problem with BrdU is its toxicity and mutagenic effects (Littlefield and Gould, 1960), and it has to be given to the animal in vivo, which gives one chance of sampling and ends in sacrifying the animal (Capuco et al., 2001). This was not possible in studies like ours with a unilateral design following the experimental units (udder halves) over the course of time (repeated measurements). Another point to be considered with regards to such techniques, is the duration of the cell cycle which is different in various cell types (Smith and Martin, 1973), and about 24 hours for mammary epithelial cells (Norgaard, 2007).

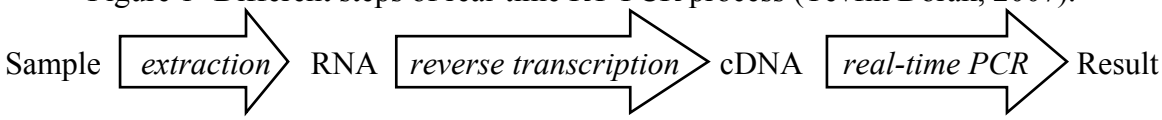
TUNEL. The duration of apoptosis in a cell is about three hours (Bursch et al., 1990; Capuco et al., 2001). The TUNEL assay is based on labelling of the 180-200 nucleotide DNA fragments, which are characteristic for apoptotic cell (Bortner et al., 1995; Norgaard, 2007). Short duration (3 hours) of apoptosis compared to substantially longer duration of proliferation (24 hours) is the reason why a direct comparison between the outcomes of the two staining methods in tissue sections can not be made.

Appendix 7

Real time RT-PCR

Reverse transcription (RT) followed by a polymerase chain reaction (PCR) is the best known technology for detection of the amounts (relative quantification) of mRNA (Lockey et al., 1998) (see figure 1). When there is a small amount of tissue (for example, biopsy sample) and with low abundant transcripts and low RNA concentrations, qRT-PCR is known as the most sensitive and most reliable method, because of its high sensitivity and potential for quantitative applications (Bustin, 2000; Freeman et al., 1999; Schmittgen et al., 2000). As shown in figure 1, it is a multi-step process; single strand RNA extraction from the tissue sample, and then, its conversion to a double-stranded complementary DNA (cDNA, which is more resistant to degradation than RNA), and then followed by its exponential amplification in a series of PCR reactions, called real-time PCR replicating the cDNA (of typically 120-200 nucleotides) for e.g. 40 times.

Figure 1- Different steps of real-time RT-PCR process (Tevfik Dorak, 2007).



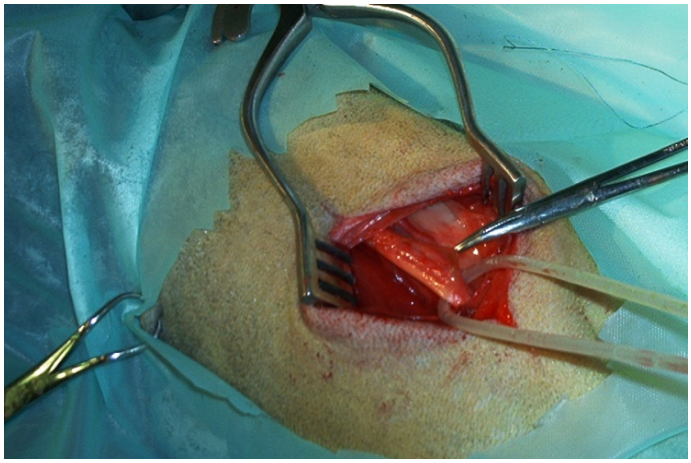
To do our analyses, two systems for real-time RT-PCR method have been used (Bustin, 2000): 1) SYBR® Green, a fluorescent dye binds to cDNA and becomes fluorescent. and 2) TaqMan® , which is based on binding of a specific fluorescent probe to the template between the two primers. Extension of primers displaces and cleaves the probe. When cleaved, the probe becomes fluorescent. The second method results in a high PCR specificity and provides strong assays even with poor quality samples. As much as possible, the TaqMan® method was prioritized to be used. Respect to shortages at the time (in year 2006), such as availability of designing software as well as time and economical limitations, the other method, SYBR® Green was chosen.

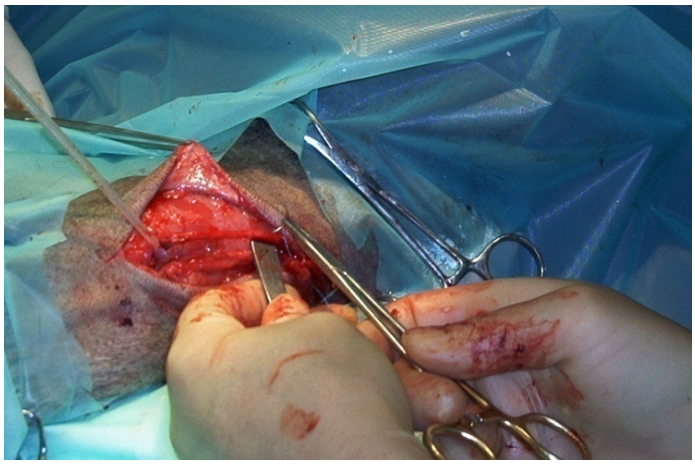
Being built on continuous amplification of the target gene, the main positive point for real-time RT-PCR method is its high sensitivity to low expressed genes. In general, real-time RT-PCR as a fairly fast and cheap method, made it possible and realistic to look for many biological patterns in our study.

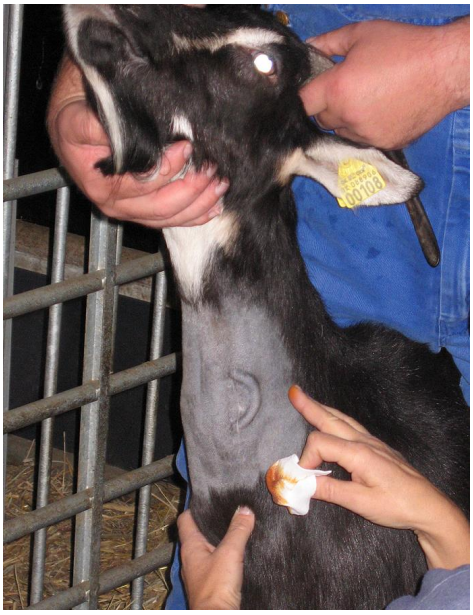
Appendix 8

Techniques for subcutaneous replacement of the carotid artery (Fleet and Mepham, 1983)

- Cut the skin dorsally and parallel to the direction of the jugular vein.
- Loosen the skin from the facia if the facia is not cut through along the incision.
- Using non-traumatic dissection, find the artery behind the jugular vein and between the muscles. It runs in the tunica together with vagus nerve.
- Loosen the tunica with both artery and nerve from the underlying tissue
- Using a rubber band, lift both carotid artery and the nerve (figure above).
- Free the artery from the nerve.
- Leaving the nerve in its original place, reclose the facia with absorbable sutures underneath the freed artery (figure in front).
- Using 2-3 single interrupted sutures (4-0 absorbable suture and and a non-traumatic needle), fix the artery to the subcutaneous layer in an arch above the incision
- Close the incision using first a subcutaneous continuous absorbable suture in the subcutaneous to fixate the wound and then single interrupted monofilament sutures in the skin.





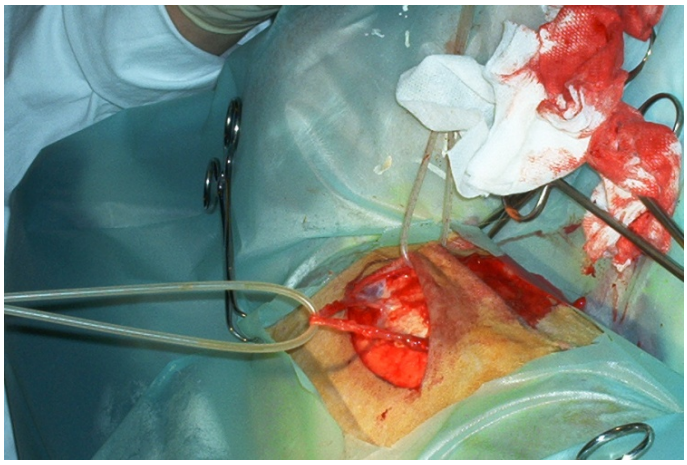


Appendix 9

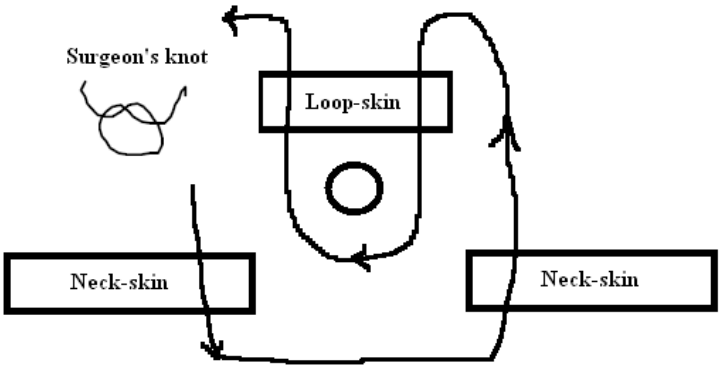
Techniques for making skin-loop around the milk vein (Fleet and Mepham, 1983)

- Make two parallel incisions (about 7.5 cm each) with a distance of about 4.5 cm both dorsal and ventral parts around the vein (figure below).

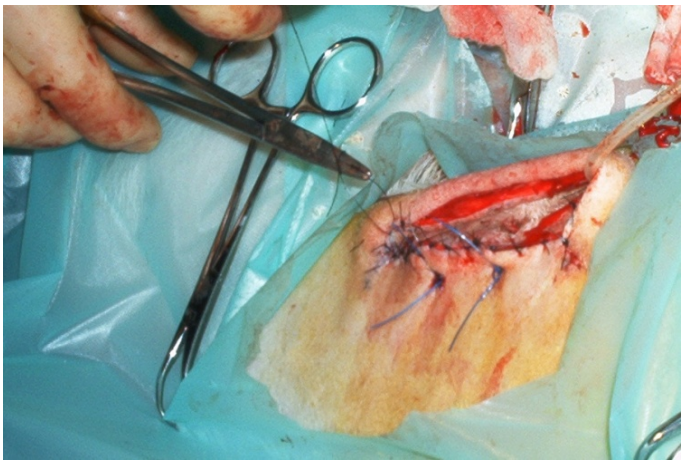
- Dissect the skin from the underlying tissue and the vein.
- Using 1-2 rubber band(s), elevate the skin (figure below).
- Dissect the vein from the underlying tissue.
- Using 1-2 rubber band(s), elevate the vein (figure below).



- Use two vertical U-sutures (0 monofilament suture) to draw the abdominal skin edges towards one another. It makes it easier to do the final suturing.
- Suture the loop and the abdominal skin (3-0 monofilament suture).
- Using just one assembling suture on each end, close the wound edges of the loop-skin and wound edges of the neck-skin (figure below)



- Suture (single interrupted) the abdominal skin (figure below).



- Suture (single interrupted) the the loop-skin around the vein.

Appendix 10

Catheterisations (for continuous infusion)

In the continuous infusion project, temporary home-made catheters (Portex translurent PVC, 0.63 ID, 1.40 OD, Smiths SIMS Portex limited, UK) were inserted in one *Vena Jugularis* prior to the start of infusing the solutions. To prepare the catheter, the material was cut in pieces to a length of 40 cm and marks were made with a speed marker 10, 15 and 20 cm from the one end. They were placed in a hibitane-ethanol solution (70% ethanol) for disinfection. The animal was prepared on the neck by shaving, washing and disinfecting with iodine. The place of insertion, on top of *Vena Jugularis* was marked with a speed marker and 1 ml lidocain (local anaesthesia) was applied under the skin. After a few minutes, a 3 inches 14G needle was introduced into the vein and the catheter was pushed through the needle, which was then removed. The depth of the catheter was adjusted according to the marks, fitted with a rubber band at the exit point from the skin, which was then sutured to the skin. The catheter was placed in a pouch and the animal was fitted with a protecting collar. Temporary pre-made catheters were inserted in *Vena Jugularis* (Secalon® T ,18 G, Ohmeda, Swindon, UK) and into an exteriorised milk vein (*v. Caudalis Superficialis Epigastrica*) (Venflon®, 20G, Ohmeda, Swindon, UK). The place of insertion, on top on *Vena Jugularis* was marked with a speed marker, and 1 ml lidocain (20mg/ml; local anaesthesia) was applied subcutaneously. A small incision was made through the skin and the underlying fascia, and thereafter the catheter was introduced. The needle was removed and the *Vena Jugularis* catheter was sutured to the skin, whilst the milk vein catheter was fitted with sleek around the milk vein loop. The function of the catheter was checked, and the catheter was rinsed with 5 ml isotonic NaCl solution containing 100 IU /ml heparin to avoid clotting of blood in the catheter in between samplings. The catheter was placed in a protecting collar of Danagrip and Co-Plus® (BSNmedical Ltd, BB9 5NJ, USA) around the neck and body of the animal. Catheters were flushed with a heparin solution (100 IU/ml in saline) after each sampling to avoid clotting of blood in the catheter. Stronger solutions (e.g. 500 IU/ml) were used when the catheter was not used for longer time intervals (usually not more than two weeks).

PAPER 1

Continuous Lactation Effects on Mammary Remodelling During Late Gestation and Lactation in Dairy Goats

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Journal of Dairy Science, In Press (doi:10.3168/jds.2009-2507).

ABSTRACT

The present study aimed to 1) elucidate whether continuous milking during late gestation in dairy goats negatively impacts mammary remodelling and hence milk production in the following lactation, and 2) identify the regulatory factors responsible for changes in cell turnover and angiogenesis in the continuously lactating mammary gland.

Nine multiparous dairy goats were used. One udder half was dried-off approximately 9 weeks pre-partum (normal lactation; **NL**), and the other udder half of the same goat was milked continuously (continuous lactation; **CL**) until parturition, or until the half-udder milk yields had dropped to below 50 g/d. Mammary biopsies were obtained from each udder half just before the NL gland was dried off (**BDP**; before dry period), within the first 2 weeks after drying-off (**EDP**; early dry period, samples available only for NL glands), in the mid-dry period (**MDP**), within the last 2 weeks prior to parturition (**LDP**, late dry period), and at days 1 (the day of parturition), 3, 10, 60, and 180 of lactation. Mammary morphology was characterised in biopsies by quantitative histology, and cell turnover was determined by immunohistochemistry (TUNEL and Ki-67). Transcription of genes encoding factors involved in mammary epithelial cell (**MEC**) turnover and vascular function was quantified by quantitative reverse transcription PCR. Results demonstrated that omitting the dry period was possible in goats, but not as easy as claimed before. MEC renewal was suppressed in CL glands, which results in a smaller MEC population in the subsequent lactation. At the time of parturition (and throughout lactation), the mammary glands subjected to CL had smaller alveoli, more fully differentiated MEC and a substantially larger capillary fraction compared with NL glands. The continuously lactating gland thus resembled a normally lactating gland in an advanced stage of lactation. None of the studied genomic factors could account for these treatment differences. The described characteristics in CL glands compared to NL glands indicated a gland maintained in lactation for a longer period of time.

Key words: continued milking, cell turnover, angiogenesis, dry off

INTRODUCTION

Milk production in ruminants is determined not only by the genetic capacity of the animal but also by management factors such as duration of the dry period in between successive lactations. The dry period appears to be essential for dairy animals to ensure that the mammary epithelial cell (**MEC**) population can be effectively renewed as one lactation comes to the end and prior to onset of the next lactation (Capuco et al., 1997). The MEC population renewal is reported to be crucial to ensure optimal milk production in the following lactation in dairy cows (Madsen et al., 2008; Remond et al., 1997; Swanson, 1965). Only few studies have been performed on continuous lactation (**CL**) in dairy goats, and the impact of omitting the dry period on milk production in the next lactation in this species appears to be less clear. Fowler et al. (1991) and Mackenzie (1967) reported that CL had no negative effect on milk yield in the subsequent lactation. However, the experimental design by Fowler et al (1991) included an unusually long dry period of 24 weeks. In contrast to this, Caja et al. (2006) reported decreases in milk production of 29% in dairy goats in response to omitting the dry period.

The level of milk production and the changes in milk yield over the course of lactation, depend on three main factors: 1) the number of MEC, which in turn is affected by the balance between the rate of epithelial cell proliferation and apoptosis (Capuco et al., 2003; Knight, 2000); 2) the secretory activity of these cells, which in turn is affected by their differentiation (Akers et al., 2006); and 3) the provision of nutrients and removal of metabolic waste products via the blood.

It has been reported previously in the dairy cow that omitting the dry period and milking of dairy cows continuously from one lactation into the next will interfere with MEC cell renewal (Annen et al., 2008; Capuco et al., 1997; Sorensen et al., 2006). This is likely to provide some of the explanation for the negative impact of CL on milk production in the next lactation. It remains to be established how and by which mechanisms continuous milking during late gestation can interfere with the regulation of MEC renewal.

Angiogenesis is the process by which new capillaries are formed from pre-existing blood vessels; a process known in other species to be associated with mammary remodelling (Matsumoto et al., 1992). The mammary microvasculature indirectly plays a very important role for milk synthesis by providing the nutrients and oxygen to the MEC and removing the metabolic waste products, which is essential to sustain milk synthesis (Djonov et al., 2001). However, there is a scarcity of information available for dairy animals regarding the normal

remodelling of the mammary microvasculature during the dry period in preparation for the next lactation (Akers, 2002). It is unknown whether continued milking during the late gestation period will interfere with this remodelling of the mammary microvasculature.

We hypothesized that omission of the dry period can interfere not only with key regulatory mechanisms responsible for renewal and differentiation of the MEC population (cell turnover and lactogenesis), but also with mammary vascular function and angiogenesis, which prevents the MEC population from fully expressing their production potential. We further hypothesized that dairy goats would be similar to dairy cows in this respect.

The objectives of the present study on the dairy goats were therefore to:

1) determine if the negative impact of CL on mammary synthetic capacity in the following lactation can be ascribed to interference exclusively with mammary cell turn-over and differentiation, or whether angiogenesis and hence tissue structures supporting metabolic activity are also affected.

2) identify through which regulatory mechanisms CL interferes with mammary cell turn-over (apoptosis and cell proliferation), vascular function and angiogenesis.

MATERIALS AND METHODS

Experimental Animals

Nine Danish crossbred Landrace-Saanen dairy goats (parity ≥ 2) were used over one (5 goats) or two consecutive (4 goats) lactation periods. They were fed a diet consisting of hay ad libitum, supplemented with barley, concentrate, and molasses, according to their requirements. Animals had free access to fresh water and a vitamin/mineral supplement. They were milked manually at 0900 and 1530 h and fed twice a day at 0730 and 1430 h, half the ration being given at each feeding. The experiment was carried out at the experimental facilities at the Faculty of Life Sciences, University of Copenhagen, Denmark, and approved by the Danish Animal Experimentation Inspectorate, and complied with the Danish Ministry of Justice laws concerning animal experimentation and care for experimental animals.

Experimental Design

The experimental design was a randomized complete block design with two treatments in 18 blocks. The two udder halves in each animal were randomly subjected to two different treatments, thus using the animal as its own control: one udder half was dried-off approximately 9 weeks prior to expected kidding followed by a normal lactation (NL),

whereas the other udder half was milked continuously throughout pregnancy and into the next lactation (CL). Omission of the dry period was not done in the same gland two consecutive years. Milk yield of individual udder halves was recorded daily during the first 10 days of lactation, and twice per week during the rest of the experiment.

Mammary Gland Biopsies

Mammary biopsies were obtained from both mammary glands at different time points during gestation and lactation: just before drying-off of the NL gland (before dry period; BDP), within the first 2 weeks after drying-off (early dry period; EDP) (samples available only for NL glands), in the mid-dry period (MDP), within the last 2 weeks prior to parturition (LDP, late dry period), and at d 1 (day of parturition), 3, 10, 60, and 180 of lactation (Figure 1). From each mammary gland, two biopsies were sampled according to the procedure described by Cvek et al. (1998).

Histology and immunohistochemistry. Mammary biopsies were fixed in 4% paraformadelhyde (**PFA**), embedded into paraffin blocks, sliced and prepared to be stained for detection of cell apoptosis (TUNEL) and proliferation (Ki67), and histomorphological studies (H&E and PAS), as described by Norgaard et al. (2008a). Random micrographs were taken at primary magnification 400x at a light microscope (Leica DMR, Leica microsystems, Germany) with a camera (Leica DPC 490, Leica microsystems, Germany) connected to computer. Using ImageJ software (Abramoff et al., 2004), a 30 points transparent grid was put on each micrograph. Proportions refer to the number of point hits on a given cell or tissue structure compared to the total number of hits, and numbers refer to the total number of that structure observed in the whole picture. When a point was hitting a mammary epithelial cell (MEC), it was classified into poorly, intermediate or fully differentiated epithelial cell according to its secretory activity as described by Akers et al. (2006). Briefly, a fully differentiated polarized MEC has a basally located and rounded nucleus, a high cytoplasm to nucleus ratio, and many vacuoles including large apical lipid droplets. Intermediately differentiated MEC have fewer vacuoles, more irregularly shaped nuclei, and a smaller cytoplasm to nuclei ratio compared with fully differentiated cells. Poorly differentiated MEC have very few if any vacuoles, large or randomly positioned lipid droplets, very little cytoplasm, and an oval (flattened) nucleus (Figure 2). The counting of coinciding points with the mentioned desired structures allowed the unbiased estimation (Gundersen et al., 1988) of volume fractions for each animal.

Real Time Reverse Transcription-PCR. Genes were chosen to be analyzed based on their suggested roles in mammary growth, remodelling and function (Table 1). As shown in tables 1-3, target genes were divided in two groups: 1) Genes related to vascular function: cyclooxygenase I (**COX1**), cyclooxygenase II (**COX2**), prostocylcine synthase (**PTGIS**), thromboxane A2 synthase (**TBXAS**) and carbonic anhydrase IV (**CA4**), and angiogenesis: angiopoietin I (**ANGPT1**), angiopoietin II (**ANGPT2**), tyrosine kinase tie2 receptor (**RTK**), vascular endothelial growth factor (**VEGF**), vascular endothelial growth factor receptor 1 (**VEGFR1**) and vascular endothelial growth factor receptor 2 (**VEGFR2**), and 2) Genes related to cell turnover and lactogenesis: B-cell CLL/Lymphoma- 2 (**BCL2**), Bcl2-Associated X Protein (**BAX**), cyclin D1 (**CCND1**), insulin-like growth factor I (**IGF1**), insulin-like growth factor I receptor (**IGF1R**), insulin-like growth factor binding protein III (**IGFBP3**), insulin-like growth factor binding protein V (**IGFBP5**), transforming growth factor beta I (**TGFB1**), transforming growth factor beta I receptor I (**TGFB1R1**), transforming growth factor beta I receptor II (**TGFB1R2**), prolactin receptor (**PRLR**), alpha-lactalbumin (**LALBA**), lactoferrin (**LTF**), leptin (**LPT**) and leptin receptor (**LPTR**). Transcription of target genes was quantified by real time RT-PCR. Approximately 10 mg of mammary tissue was homogenized in 350 μL of RNeasy lysis buffer and diluted (1:1) with 70% ethanol. The RNA was purified using the RNeasy mini kit (Qiagen, Albertslund, Denmark) and reverse-transcribed with oligo-dT and Superscript II RNAse H reverse transcription kit (Invitrogen, Taastrup, Denmark) according to the manufacturer's protocol. Reverse-transcribed material (1 μL) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden) using gene-specific primers. Locked nucleic acid (**LNA**) probes from the human Universal ProbeLibrary (Roche Applied Science, Hvidovre, Denmark), labeled with FAM fluorophore were used for detecting amplified LPT, PRL-R, BAX, CCND1, IGF1R, LTF, ANGPT2 and COX2 genes. Power SYBR-Green PCR Master Mix (Applied Biosystems, California, USA) was used for detection of the remaining genes: LPT-R, BCL2, IGF1, IGFBP3, IGFBP5, LALBA, VEGF, VEGF-R1, VEGF-R2, CA4, TBXAS, TGFB1, TGFB1-R1, TGFB1-R2, RTK, ANGPT1, COX1 and PTGIS. Melting curves showed no more than a single amplified product. The PCR amplification signal was detected using an ABI PRISM 7900 detection system (Applied Biosystems). Transcription of glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), 18S ribosomal RNA I (**18S RNR1**) and beta-actin (**ACTB**) were quantified and their appropriateness as house keeping gene (**HKG**) were evaluated against the total RNA content. Transcription of target genes were normalized by the mean transcription level of GAPDH as HKG, as suggested by Vandesompele et al. (2002) and

described by Theil et al. (2006). The oligonucleotide sequences for the genes were designed using the Primer Express software, version 2.0 (Applied Biosystems Inc, CA, USA) or Beacon Designer Version 7.00 software (Premier Biosoft International, CA, USA). No amplification was found in ribonuclease free water and in samples of genomic bovine DNA. For qRT-PCR, 40 cycles were used at 95°C for 15 s, and 60°C for 60 s. The response was quantified as the number of PCR cycles required to reach a certain threshold, and samples were analyzed in duplicates. The oligonucleotide sequences of forward primers, LNA probes (when used), and reverse primers for the genes are shown in table 3.

Gene expression data were obtained as Ct values (the cycle number at which PCR cycles logarithmic plots cross a calculated threshold line) to evaluate mRNA quantities according to the manufacturer's guidelines, and used to determine ΔCt values:

$$\Delta\text{Ct} = [\text{Ct of the target gene}] - [\text{Ct of the reference gene (GAPDH)}]$$

Least square means of ΔCt values of target genes were normalized to the level observed at BDP of NL glands, by calculating the $\Delta\Delta$Ct values:

$$\Delta\Delta\text{Ct} = [\Delta\text{Ct observed at a given stage for CL or NL}] - [\Delta\text{Ct observed at BDP for NL}]$$

The relative mRNA quantity was calculated as $Q^{-\Delta\Delta Ct}$, where Q is 1 + PCR efficiency as determined by $10^{-1/\text{slope of standard curve}}$ -1 (Rasmussen, 2000). In cases with 100% PCR efficiency, this formula was simplified to the following:

$$\text{Relative mRNA quantity (100\% efficiency)} = 2^{-\Delta\Delta Ct}$$

All statistics were performed at the ΔCt level (Theil et al., 2006) to exclude potential bias because of averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). Expression of both 18S rRNA and ACTB but not GAPDH were affected by parity. Therefore, GAPDH was selected to be used as housekeeping gene (HKG) for data normalization.

Statistical Analysis and Calculations

Statistical evaluation of all data was performed using the Mixed procedures in SAS (SAS Institute, 2003). Variables in the statistical models included the experimental year, treatment (CL or NL), experiment day at which the biopsy sample has been taken and its interaction with treatment as fixed effects, and the factors goat, goat within experimental year, and gland within goat at each experiment day as random effects, as shown in the following model:

$$Y = \mu + \alpha_i + \beta_j + \gamma_k + \beta\gamma_{jk} + \rho_l + \tau_{il} + \upsilon_{klm} + \varepsilon_{ijklm}, \text{ where}$$

Y= the dependent variables, μ= the overall means, α_i= experimental year (i=1,2), β_j= lactation regimen (j= CL and NL), γ_k= experiment day (k= BDP, MDP, EDP, D1, D3, D10, D60 and D180), $\beta\gamma_{jk}$ = interaction between lactation regimen and experiment day, ρ_l= random effect of goat (l=1, 2,..,9), τ_{il} = random effect of goat within a year, υ_{klm} = random effect of udder half within a goat within a year (m= left, right), ε_{ijklm}= random variation which was assumed to be normally distributed with a variance σ^2 and a mean of zero. For analysis of milk yield, week of lactation was included as fixed effect instead of experimental day. Before performing the final statistical analyses, data were checked for outliers based on residual plots. The data from poorly differentiated MEC as well as cell proliferation and apoptosis were arc-sinus transformed to meet statistical assumptions for normal distribution. However, the back-transformed data are presented in figures. Presented results are expressed as least squares means with standard error of mean. The PDIFF option in SAS was used to generate comparisons between treatment means. The level of significance was set at $P < 0.05$.

RESULTS

Milk Yield

In experimental year 1, 6 glands (6 goats, and one of each) were attempted to be milked continuously during the late gestation period, but only 2 glands completed a CL with a milk production above 50 g/d until the day of parturition, In experimental year 2, out of 7 glands (7 goats, and one of each) assigned to the CL treatment, only 4 glands remained lactating throughout the entire gestation period. Thus, a total of 6 glands completed a CL out of 13 attempts over the 2 years.

Half-udder milk yield data were available only for the first 2 weeks of lactation and again from lactation day 60 and onwards due to unfortunate erroneous recordings in the period in between. However, based on these milk yield data, it was evident that out of the total number of 6 glands which completed a CL, milk production was numerically suppressed in 2, it was unaffected in 2 and was numerically increased in 2 compared with the milk production in the contra-lateral NL gland, and overall CL thus had no significant impact on milk yield (data are not shown).

Mammary morphology

Gross morphological changes. In mammary sections, the highest number of alveoli per picture (5.6), the lowest proportion of MEC (34%), lowest proportion of lumen (17%), the highest proportion of interstitial tissue (49%) and the peak in micro-vessel proportion (19%) were observed in the middle of the dry period (MDP) (Figure 2), compared to other developmental stages examined. In general, no statistical differences were observed between the CL and NL glands. Closer to parturition in period LDP, the proportion of micro-vessels (17%) remained high, alveoli proportion (66%) as well as alveoli lumen (30%) and MEC fractions (37%) increased in both CL and NL glands compared to MDP, whereas the proportion of interstitial tissue (34%) and alveoli numbers per picture (3.6) decreased. At parturition, the proportion of MEC had increased even further (44%), while lumen and interstitial tissue (30%) remained at the same high level (25%) as in LDP, and the micro-vessel proportion was decreased (12%). Overall, this resulted in peak values in alveoli proportion in sections at D1 and D10 (71 and 73%, respectively), but distributed on a lower number of alveoli. NL glands tended (P=0.08) to have a lower number of alveoli (2.8) compared to CL (3.8) glands at the day of parturition (D1).

Alveolar lumen and interstitial tissue areas remained relatively constant during early to mid-lactation, but the alveoli and MEC fractions gradually decreased. MEC decreased the most in CL glands and were similar in NL glands in mid (38 vs. 44%, respectively) and late lactation (37 vs. 41%, respectively). The micro-vessel proportion did not change in early-mid lactation in the NL glands, but increased during this period and became significantly highest from D10 and onwards in the CL glands. Concurrently, alveoli number remained at the same level in NL glands during lactation; but gradually declined in CL glands to lower levels than observed in the NL glands.

The lowest values during lactation for epithelial (39%), lumen (23%) and alveoli fractions (63%) were observed during late lactation. The decreases from early to mid-lactation were numerically more pronounced in the NL glands than in the CL glands, and alveoli number remained similar to CL (2.2) compared to NL glands (3.0) (P=0.18). The opposite was observed for interstitial tissue, where the highest values during lactation were observed in late lactation (38%), and the micro-vessel fraction was substantially higher in CL compared to NL glands (20% and 11%, respectively) at this time point.

Differentiation of MEC. As shown in Figure 2, intermediate differentiated MEC proportion was high (83%), while the poorly differentiated MEC was low (11%) in both kinds of glands in period BDP (before drying off the NL gland). At MDP, the proportion of intermediate

differentiated MEC declined to its lowest level (31%), while poorly differentiated MEC peaked (68%) at this time-point. Although poorly differentiated MEC were significantly higher in NL than CL glands at MDP (68% and 48%, respectively, P=0.01) and LDP (41% and 15%, respectively, P=0.004), the proportion of poorly differentiated MEC reached the same low level in both glands at parturition (4%) and during the following lactation. On the other hand, the proportion of intermediate differentiated MEC rose in both glands to reach a plateau (79%) a few days after parturition (D3) and remained at this level during the rest of lactation. Fully differentiated MEC proportion was low in both glands in BDP (14%), rose at LDP where it tended (P=0.10) to be higher in CL (36%) than NL glands (20%), and it remained higher in CL compared to NL glands throughout lactation, either numerically or statistically. Across the whole gestation-lactation period, CL glands thus had significantly higher (P=0.004) proportion of fully differentiated MEC (24%) than NL glands (15%); but a lower proportion of poorly differentiated MEC compared with NL glands (16% vs. 21%, P=0.003).

Immunohistochemistry

As shown in figure 3, MEC proliferation happened predominantly in late gestation prior to parturition, and was high in MDP (3.1% in CL and 6.8% in NL glands) and peaked in LDP (5.0% in CL and 8.0% in NL glands). The fraction of apoptotic cells (cell staining positive in the TUNEL assay) peaked in both glands (0.4%) in the early stage of lactation (D10). The proportion of cell staining positive for apoptosis and proliferation remained low throughout the rest of the lactation period.

In general, CL glands had lower rate of cells staining positive for proliferation than NL glands (P=0.03), particularly during gestation (MDP, P=0.001 and LDP, P=0.02). Postpartum, the CL glands tended to have higher rate of apoptosis a few days after parturition compared with the NL glands (D3, P=0.12), and hence generally lowered rates of cell renewal in the peri-partum period.

Gene Expression

Genes related to Cell turnover and lactogenesis. In general, BCL2, CCND1, IGF1, IGFBP3 and LPTR had a high level of expression in mid to late dry period, as shown in Figure 4-A. Their expression levels dropped at parturition and remained low or decreased during the remaining part of lactation. An opposite pattern was observed for LALBA which was very low prepartum and increased at parturition and remained high thereafter. Although the general

pattern of changes in gene expression was the same in the two experimental years, the expression of LALBA was for unknown reasons higher in the second experimental year than the first.

CL glands had significanlty higher expression of LALBA (P=0.03) compared to NL glands, and this was due to higher expression in CL glands of LALBA in late gestation, where CL glands were lactating and NL glands dried off . Contrast analyses, performed at each sampling stage, showed that CL glands compared to NL glands had higher mRNA expression of LTF (P=0.01) in LDP, CCND1 (P=0.04) at parturition, and of BAX in late lactation (P=0.03). In addition, there was a tendency for higher expression of IGFBP5 (P=0.08) at parturition and of IGF1 in mid lactation (P=0.08). In late lactation, however, expression of LTF was found to be lower (P=0.01) in CL compared to NL glands. There were no significant changes in expression of IGF1R, TGFB1, TGFB1R1, TGFB1R2, PRLR and LPT (data not shown) during the course of gestation-lactation in neither CL nor NL glands and no impact of CL on expression of these genes.

Genes related to vascular function and angiogenesis. As shown in Figure 4-B, expression levels of CA4 and VEGF were low in LDP, increased at parturition and peaked a few days after (D10), and thereafter remained the same in CL than NL glands. Expression of ANGPT1 and COX2 was high during the dry period, especially at MDP in NL glands and at parturition day in CL glands. ANGPT1 decreased to a lower level a few days after parturition (D3) and remained constant until late lactation. With a little delay, COX2 started to gradually decrease from D10 of lactation and reached its lowest level in mid-late lactation. For the rest of the studied genes (COX1, PTGIS, TBXAS and RTK), expression levels were fairly constant throughout the course of gestation-lactation (data not shown). The general pattern of changes in gene expression was the same in the two experimental years, but expression of ANGPT2 was higher in the second year.

In general, CA4 (P=0.06) tended to be higher in CL than NL glands. CL glands had higher expressions compared to NL glands of ANGPT1 (P=0.002) and VEGFR2 (P=0.007) at parturition, and tended to have higher expressions also of CA4 (P=0.08) at D10. However, expressions were lowest for VEGFR1 (P=0.02) at D3 and tended to be lowest also for ANGPT2 (P=0.07) at D180 compared to NL glands.

DISCUSSION

Continuous Milking and Milk Production in Dairy Goats

Although earlier reports (Fowler et al., 1991; Mackenzie, 1967) have claimed that goats are more capable of maintaining milk production throughout gestation when being continuously milked compared to cows, we, similar to Caja et al. (2006) experienced considerable problems in preventing the glands from drying off spontaneously in very late lactation. We therefore believe that goats and cows are more similar than it has been postulated in earlier studies (Fowler et al., 1991; Mackenzie, 1967) with respect to their ability to maintain milk production throughout the very late gestation period. In the glands that managed to lactate continuously throughout late gestation, the consequences for milk production in the next lactation, however, appeared to be far less severe than reported for dairy cows (Annen et al., 2008; Madsen et al., 2008; Swanson, 1965), and thus in most goats milk yield may not be negatively affected at all. Irrespective of any impact on subsequent milk production, CL clearly does not prevent lactogenesis. This has been demonstrated in several other studies in cows as well as in dairy goats by the increase in milk yield right after parturition (Annen et al., 2008; Caja et al., 2006; Fowler et al., 1991), and further documented in this study by the clear and similar increase in expression of LALBA in CL and NL glands.

Impact of CL on Mammary Epithelial Cell Remodelling

Consistent with other studies in cow (Norgaard et al., 2005), sheep (Norgaard et al., 2008a) and goat (Caja et al., 2006), our results confirmed that the major part of mammary redevelopment takes place in late pregnancy. At parturition, the proportion of poorly differentiated MEC was decreased, whilst the proportion of particularly intermediate differentiated MEC was increased. It is reasonable to suggest that the poorly differentiated MEC are the newly formed cells in the mammary gland, that need to differentiate further to acquire lactational ability. CL glands had a 3-7% lower proportion of MEC in the subsequent mid-late lactation compared to NL glands, due to lower proliferation rate during MDP-LDP.

Milk yield turned out to be virtually unaffected by CL in the glands that were capable of completing a continuous lactation, despite the negative impact of CL on MEC renewal. CL glands, however, had a higher proportion of more fully differentiated MEC throughout lactation, and this indicates that the fully differentiated, and presumably older, MEC possesses higher synthetic capacity compared to the intermediately differentiated MEC. It would be relevant to find out how regulation of mammary remodelling differs between cows

and goats and thus find the explanation for why the yield in the CL goats is not affected as it is in cows milked continuously. We suggest that the MEC in goats survive longer than in cows.

In our histological samples, we observed that MEC undergoing proliferation occurred predominantly in the intermediately differentiated rather than in the poorly and fully differentiated MEC (69%, 23% and 9%, respectively). We found no apoptotic cells among poorly differentiated MEC, but only in the intermediately or fully differentiated MEC. However, due to the disappearance of cell borders in our TUNEL slides, it was difficult to make a clear distinction of the intermediately from the fully differentiated MEC. Moreover, there were no differences in these relative distributions between the CL and NL glands. It is therefore puzzling why CL glands cannot restore their MEC population in late gestation to the same extent as in the NL glands, when the CL glands apparently possess MEC in a stage of differentiation that are capable of undergoing both cell proliferation and initiation of lactogenesis (see below) just as in NL glands.

Impact of CL on Vascular Remodelling

Very few studies have addressed the changes in mammary vasculature during the normal course of gestation-lactation in ruminants, and to our knowledge no previous studies have looked into the impact of continued milking during late gestation on vascular remodeling. We found that in the goat mammary gland, the vessel fraction in histological slides peaked earlier in the NL glands (MDP) compared to the CL glands (LDP), and thereafter the vessel fraction decreased to the lowest levels in early lactation. This is consistent with the higher rate of formation of new cells in the NL glands throughout the dry period, resulting in the vasculature gradually occupying less and less space. Omission of the dry period resulted in an either numerically or statistically, higher proportion of micro-vessels in the following lactation. Thus, the results may indicate that the larger ratio of capillaries-to-alveoli in CL compared to NL glands is indicative of a greater blood supply to the MEC and more efficient exchange of nutrients and waste products across the capillary-MEC barrier in CL glands. This could partly explain why milk yield in CL glands in goats are unaffected by CL despite the negative impact of CL on pre-partum cell renewal. However, the capillaries-to-alveoli ratio will normally be at its lowest in early lactation and increase as lactation progresses (M. O. Nielsen, Faculty of Life Sciences, University of Copenhagen, Denmark; K. Dahlborn and K. Cvek, Department of Anatomy, Physiology and Biochemistry, The Swedish University of Agricultural Science, Sweden; present study). It is thus inversely related to mammary blood

flow changes (Nielsen et al., 1990) and efficiency of nutrient extraction in the mammary gland during lactation (Nielsen et al., 2001). In fact, the number of capillaries surrounding each alveolus is several fold higher in the early compared to the late lactation, but the capillary diameter is very small in early lactation and increases as lactation progresses (M. O. Nielsen, Faculty of Life Sciences, University of Copenhagen, Denmark; K. Dahlborn and K. Cvek, Department of Anatomy, Physiology and Biochemistry, The Swedish University of Agricultural Science, Sweden). Very small capillaries would favor a more efficient nutrient exchange across the capillary – MEC barrier due to smaller diffusion distances, and extraction rates for a number of nutrients have in fact been found to be higher in early compared to late lactation (Madsen et al., 2008; Nielsen et al., 2001). Our findings therefore indicate that CL glands enter the new lactation with an older population of MEC surrounded by a similarly older (and possibly more developed) capillary network. As a result, nutrient exchange across the capillary-MEC barrier would occur less efficiently, but the decrease in vascular resistance (larger peripheral resistance) could possibly allow for a compensatory increase in mammary blood flow. Future studies will need to be conducted to reveal if milk yield in this way could be maintained relatively unaffected by CL in the goat due to a higher mammary blood perfusion per unit of milk synthesized.

Impact of CL on Key Regulatory Mechanisms Responsible for MEC Population

We were not able to demonstrate any significant effects of CL on expression of any of the genes encoding for factors with reported effects on cell turnover and differentiation (Figure 4A). Contrast analyses revealed differences in expression between CL and NL glands for a few genes at selected time points. CL glands had higher expressions in period LDP of LTF gene, and at parturition (D1) of CCND1 and IGFBP5 genes. LTF is an innate immune factor and reported to be increased during involution compared to lactation. LTF expression in general did not change during the lactation period, and the higher expression of this mitogenic factor (Baumrucker et al., 2006) in CL glands in LDP was not consistent with the fact that CL glands had much lower rates of cell proliferation in late gestation compared to NL glands. CCND1, BCL2, IGF-1 and leptin have all been reported to have mitogenic effects on MEC (Baumrucker and Erondu, 2000; Dlugosz et al., 2006; Sherr, 1995). Expression of CCND1, BCL2 and the LPTR increased across the pre-partum period, and decreased at parturition to reach the lowest levels during lactation. The same was reported in dairy cows (Annen et al., 2007) and sheep (Norgaard et al., 2008a). These expression changes followed the general changes in MEC proliferation. However, CL did not affect expression of any of these factors

at any time point during gestation or lactation (except the higher expression for CCND1 at D1 in CL glands) and could therefore not account for the negative effect of CL treatment on MEC renewal.

BAX and IGFBP5 are potent apoptotic factors in the mammary gland (Sorensen et al., 2006; Wareski et al., 2001). BAX expression followed the same general pattern as observed for the mentioned mitogenic agents with highest expression pre-partum. As others before us (Norgaard et al., 2008b; Wareski et al., 2001), we have not been able to correlate BAX gene expression to the changes in apoptosis during the transition period from gestation to lactation and the peak observed in the early postpartum period (D3-D10). IGFBP5 was highly expressed at D1 in CL glands, and CL glands happened to have a close to significantly higher rate of apoptosis on D3 compared to NL glands. Upregulation of IGFBP5 around parturition may be a mechanism whereby cell death is induced in early lactation, whereby the oldest MEC remaining from the previous lactation can be renewed in CL glands. Another finding in support of this proposition was that the higher rate of apoptosis in CL glands at D3 indeed coincided at this exact time point with a drop in the proportion of fully differentiated MEC, which was not observed in NL glands.

The factors or mechanisms explaining the impact of CL in late gestation on cell renewal and differentiation thus still remain to be identified. However, our failure to link the cell turnover events to changes in gene transcription could also be due the fact that these expression changes are cell specific. When performing expression studies on tissue samples consisting of several different cell types, as in mammary tissue, cell specific changes in gene expressions could have been masked, or might have been diluted at stages where the proportion of MEC was low. Obviously the impact of continued milking in late gestation on cell renewal and mammary remodelling must be mediated by local mechanisms in the gland subjected to CL, since it is possible to continuously milk one udder half in the animal whilst drying-off the other.

Effect of CL on Key Regulatory Mechanisms Responsible for Vascular Function

In contrast to the factors encoding for MEC turnover, CL did affect changes in expression during gestation and at the day of parturition for some of the genes encoding for factors regulating vascular function. Djonov et al. (2001) has suggested that regression of the capillary endothelium during mammary involution could be correlated to or be a consequence of MEC involution. Higher gene expression of CA4 (in general ($P<0.06$)and particularly in the pre-partum period) and VEGFR1 during the first part of the dry period in CL glands

maybe ascribed to the fact that those glands had a MEC population that was prevented from involution, and thus maintained into lactation. Consequently a functional capillary network was sustained as well.

Statistically or numerically higher expressions of ANGPT1, VEGF, VEGFR1, VEGFR2 and COX2 in CL glands at d1, suggest that the CL glands experiences a higher rate of vascular growth than the NL glands, probably renewing part of the ageing vascular system at parturition, whereas this vascular renewal would have happened before parturition in the NL glands in parallel with the renewal of the MEC population.

CONCLUSIONS

CL suppressed MEC renewal, because of suppression of pre-partum cell proliferation; however, the rate of apoptosis was unaffected. Continuously lactating mammary glands of goats therefore entered the subsequent lactation with a smaller MEC population, as known in dairy cows. CL glands throughout lactation had a larger micro-vessel proportion, lower proportion of MEC, but more fully differentiated and likely older population of MEC compared to NL glands. If fully differentiated MECs have higher secretory activity, this could in part explain why goats can lactate continuously without major negative impact on milk yield, in spite of the smaller population of MEC. We were not able to resolve why continued milking during the late gestation period interferes with MEC and vascular remodelling within the mammary gland, and what regulatory mechanisms are responsible. So two puzzling questions remain: Why is the full restoration of the MEC population and overall mammary remodelling inhibited just because of continuous milk removal from a mammary gland throughout gestation? And why is this associated with a marked depression in milk yield in dairy cows in the subsequent lactation, but apparently not in the dairy goat that (with some difficulty) manage to lactate continuously?

ACKNOWLEDGMENTS

This project was financed by the Danish Research Council for Technology and Production Sciences. Sina Safayi was in receipt of a PhD scholarship co-financed by the Danish Research Council and by Mrs. M. Namian. The authors would like to acknowledge V. G. Christensen, D. S. Jensen, R. Jensen, and H. A. V. Ruby, Faculty of Life Sciences, University of Copenhagen, Denmark, and K. B. Poulsen, Faculty of Agricultural Sciences, Aarhus

University, Denmark, for their valuable technical assistance. We also express our gratitude for the valuable advice in histology, statistics and stereology, respectively, from V. S. Elbrønd, C. Th. Ekstrøm and D. L. Wulfsohn, Faculty of Life Sciences, University of Copenhagen, Denmark.

REFERENCES

Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image Processing with ImageJ. Biophotonics International 11(7):36-42.

Akers, R. M. 2002. Lactation and the Mammary Gland. First ed. Blackwell publishing, Ames, Iowa, USA.

Akers, R. M. 2006. Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. J. Dairy Sci. 89(4):1222-1234.

Akers, R. M., A. V. Capuco, and J. E. Keys. 2006. Mammary histology and alveolar cell differentiation during late gestation and early lactation in mammary tissue of beef and dairy heifers. Livestock Science 105(1-3):44-49.

Allan, G. J., J. Beattie, and D. J. Flint. 2004. The role of IGFBP-5 in mammary gland development and involution. Domestic Animal Endocrinology 27(3):257-266.

Annen, E. L., A. C. Fitzgerald, P. C. Gentry, M. A. McGuire, A. V. Capuco, L. H. Baumgard, and R. J. Collier. 2007. Effect of continuous milking and bovine somatotropin supplementation on mammary epithelial cell turnover. J. Dairy Sci. 90(1):165-183.

Annen, E. L., C. M. Stiening, B. A. Crooker, A. C. Fitzgerald, and R. J. Collier. 2008. Effect of continuous milking and prostaglandin E-2 on milk production and mammary epithelial cell turnover, ultrastructure, and gene expression. Journal of Animal Science 86(5):1132-1144.

Baumrucker, C. R., and N. E. Erondu. 2000. Insulin-like growth factor (IGF) system in the bovine mammary gland and milk. Journal of Mammary Gland Biology and Neoplasia 5(1):53-64.

Baumrucker, C. R., F. Schanbacher, Y. F. Shang, and M. H. Green. 2006. Lactoferrin interaction with retinoid signaling: Cell growth and apoptosis in mammary cells. Domestic Animal Endocrinology 30(4):289-303.

Caja, G., A. A. K. Salama, and X. Such. 2006. Omitting the dry-off period negatively affects colostrum and milk yield in dairy goats. J. Dairy Sci. 89(11):4220-4228.

Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in Holstein cows during the dry period: Quantification of nucleic acids and histology. J. Dairy Sci. 80(3):477-487.

Capuco, A. V., S. E. Ellis, S. A. Hale, E. Long, R. A. Erdman, X. Zhao, and M. J. Paape. 2003. Lactation persistency: Insights from mammary cell proliferation studies. Journal of Animal Science 81:18-31.

Claria, J. 2003. Cyclooxygenase-2 biology. Current Pharmaceutical Design 9(27):2177-2190.

Conway, E. M., D. Collen, and P. Carmeliet. 2001. Molecular mechanisms of blood vessel growth. Cardiovasc Res 49(3):507-521.

Cvek, K., K. Dahlborn, and Y. Ridderstrale. 1998. Localization of carbonic anhydrase in the goat mammary gland during involution and lactogenesis. Journal of Dairy Research 65(1):43-54.

Djonov, V., A. C. Andres, and A. Ziemiecki. 2001. Vascular remodelling during the normal and malignant life cycle of the mammary gland. Microscopy Research and Technique 52(2):182-189.

Dlugosz, P. J., L. P. Billen, M. G. Annis, W. J. Zhu, Z. Zhang, J. L. Lin, B. Leber, and D. W. Andrews. 2006. Bcl-2 changes conformation to inhibit Bax oligomerization. Embo Journal 25(11):2287-2296.

Fowler, P. A., C. H. Knight, and M. A. Foster. 1991. Omitting the Dry Period Between Lactations Does Not Reduce Subsequent Milk-Production in Goats. Journal of Dairy Research 58(1):13-19.

Grill, C. J., and W. S. Cohick. 2000. Insulin-like growth factor binding protein-3 mediates IGF-I action in a bovine mammary epithelial cell line independent of an IGF interaction. Journal of Cellular Physiology 183(2):273-283.

Gundersen, H. J. G., T. F. Bendtsen, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J. R. Nyengaard, B. Pakkenberg, F. B. Sorensen, A. Vesterby, and M. J. West. 1988. Some New, Simple and Efficient Stereological Methods and Their Use in Pathological Research and Diagnosis - Review Article. Apmis 96(5):379-394.

Haughn, L., R. G. Hawley, D. K. Morrison, H. von Boehmer, and D. M. Hockenbery. 2003. BCL-2 and BCL-X-L restrict lineage choice during hematopoietic differentiation. Journal of Biological Chemistry 278(27):25158-25165.

Knight, C. H. 2000. The importance of cell division in udder development and lactation. Livestock Production Science 66(2):169-176.

Mackenzie, D. 1967. Goat Husbandry. 2nd ed. Faber & Faber, London.

Madsen, T. G., M. O. Nielsen, J. B. Andersen, and K. L. Ingvartsen. 2008. Continuous lactation in dairy cows: Effect on milk production and mammary nutrient supply and extraction. J. Dairy Sci. 91(5):1791-1801.

Matsumoto, M., H. Nishinakagawa, M. Kurohmaru, Y. Hayashi, and J. Otsuka. 1992. Pregnancy and Lactation Affect the Microvasculature of the Mammary-Gland in Mice. Journal of Veterinary Medical Science 54(5):937-943.

Nielsen, M. O., K. Jakobsen, and J. N. Jorgensen. 1990. Changes in Mammary Blood-Flow During the Lactation Period in Goats Measured by the Ultrasound Doppler Principle. Comparative Biochemistry and Physiology A-Physiology 97(4):519-524.

Nielsen, M. O., T. G. Madsen, and A. M. Hedeboe. 2001. Regulation of mammary glucose uptake in goats: role of mammary gland supply, insulin, IGF-1 and synthetic capacity. Journal of Dairy Research 68(3):337-349.

Nielsen, M. O., S. Nyborg, K. Jakobsen, I. R. Fleet, and J. Norgaard. 2004. Mammary uptake and excretion of prostanoids in relation to mammary blood flow and milk yield during pregnancy-lactation and somatotropin treatment in dairy goats. Domestic Animal Endocrinology 27(4):345-362.

Norgaard, J., A. Sorensen, M. T. Sorensen, J. B. Andersen, and K. Sejrsen. 2005. Mammary cell turnover and enzyme activity in dairy cows: Effects of milking frequency and diet energy density. J. Dairy Sci. 88(3):975-982.

Norgaard, J. V., M. O. Nielsen, P. K. Theil, M. T. Sorensen, S. Safayi, and K. Sejrsen. 2008a. Development of mammary glands of fat sheep submitted to restricted feeding during late pregnancy. Small Ruminant Research 76(3):155-165.

Norgaard, J. V., M. T. Sorensen, P. K. Theil, I. Sehested, and K. Sejrsen. 2008b. Effect of pregnancy and feeding level on cell turnover and expression of related genes in the mammary tissue of lactating dairy cows. Animal 2(4):588-594.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29(9).

Rasmussen, R. P. 2000. Quantification on the LightCycler. Rapid Cycle Real-Time PCR, Methods and Applications (eds S Meuer, CT Wittwer and K Nakagawara):21-34. Springer Press, Heidelberg.

Remond, B., J. Kerouanton, and V. Brocard. 1997. The effect of reducing or omitting the dry period on the performance of dairy cows. Productions Animales 10(4):301-315.

Riley, L. G., P. C. Wynn, P. Williamson, and P. A. Sheehy. 2008. The role of native bovine alpha-lactalbumin in bovine mammary epithelial cell apoptosis and casein expression. Journal of Dairy Research 75(3):319-325.

SAS Institute. 2003. SAS v. 9.1. in SAS Institute Inc., NC.

Sherr, C. J. 1995. D-Type Cyclins. Trends in Biochemical Sciences 20(5):187-190.

Silva, L. F. P., M. J. VandeHaar, M. S. W. Nielsen, and G. W. Smith. 2002. Evidence for a local effect of leptin in bovine mammary gland. J. Dairy Sci. 85(12):3277-3286.

Sorensen, M. T., J. V. Norgaard, P. K. Theil, M. Vestergaard, and K. Sejrsen. 2006. Cell turnover and activity in mammary tissue during lactation and the dry period in dairy cows. J. Dairy Sci. 89(12):4632-4639.

Swanson, E. W. 1965. Comparing Continuous Milking with 60-Day Dry Periods in Successive Lactations. J. Dairy Sci. 48(9):1205-&.

Theil, P. K., I. L. Sorensen, M. Therkildsen, and N. Oksbjerg. 2006. Changes in proteolytic enzyme mRNAs relevant for meat quality during myogenesis of primary porcine satellite cells. Meat Science 73(2):335-343.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3(7):research0034.

Wall, E. H., H. M. Crawford, S. E. Ellis, G. E. Dahl, and T. B. McFadden. 2006. Mammary response to exogenous prolactin or frequent milking during early lactation in dairy cows. J. Dairy Sci. 89(12):4640-4648.

Ward, P. P., E. Paz, and O. M. Conneely. 2005. Multifunctional roles of lactoferrin: a critical overview. Cellular and Molecular Life Sciences 62(22):2540-2548.

Wareski, P., T. Motyl, Z. Ryniewicz, A. Orzechowski, B. Gajkowska, U. Wojewodzka, and T. Ploszaj. 2001. Expression of apoptosis-related proteins in mammary gland of goat. Small Ruminant Research 40(3):279-289.

Table 1- Roles of the analyzed genes.

Gene*	Suggested Role(s)
Vascular Function and Angiogenesis:	
COX1 and COX2	involved in angiogenesis, regulating arterio-venous differentiation, and contributing in the production of prostaglandins and thromboxane (Claria, 2003).
PTGIS	catalyses the synthesis of prostacyclin I_2 which is a potent arterial vasodilator (Claria, 2003).
TBXAS	catalyses the synthesis of thromboxane A_2 which is a venous vasoconstrictor and affect vascular permeability (Claria, 2003), and might also have a role in regulation of mammary cell proliferation or secretory activity (Nielsen et al., 2004)
CA4	catalyses the reversible hydration of CO_2 to HCO_3^- and H^+, positively correlated with the mammary gland synthetic activity (Cvek et al., 1998)
ANGPT1	involved in maturation of blood vessels, regulates the formation and stabilization of the blood vessel network (Conway et al., 2001)
ANGPT2	ANGPT1 antagonist; destabilizes the vasculature and initiates angiogenesis in the presence of VEGF (Conway et al., 2001)
RTK	receptor for ANGPT1 ad ANGPT2 (Conway et al., 2001)
VEGF	potent angiogenic factor and possess vascular permeability-inducing properties (Conway et al., 2001)
VEGFR1	negative regulator of VEGFR-2 (Conway et al., 2001)
VEGFR2	VEGF receptor mediating the mitogenic and permeabilizing properties of VEGF (Conway et al., 2001)
Cell turnover and Lactogenesis:	
BAX	pro-apoptoticfactor (Haughn et al., 2003)
BCL2	anti-apoptotic factor (Haughn et al., 2003)
CCND1	involved in regulation of cell proliferation (Sherr, 1995)
IGF1	plays an important role as a survival factor during mammary gland development and remodelling during involution (Allan et al., 2004)
IGF1R	impacts mammary growth and inhibits apoptosis (Baumrucker and Erondu, 2000)
IGFBP3	multifunctional protein, acts either as a growth facilitator or inhibitor (Grill and Cohick, 2000)
IGFBP5	related to apoptosis of the mammary gland, and has the ability to inhibit cell proliferation (Allan et al., 2004)
TGFB1	anti-proliferative and apoptogenic factor for MECs (Wareski et al., 2001)
TGFB1-R1 and -R2	receptors for and mediating actions of TGFB1 (Akers, 2006)
PRLR	PRL receptor; upon binding to its receptor, PRL activates several pathways associated with MEC proliferation, differentiation, and lactogenesis (Wall et al., 2006)
LALBA	milk whey protein and a component of the enzyme complex regulating lactose synthesis and thus milk volume (Riley et al., 2008). Indicator of onset of copious milk secretion at lactogenesis
LTF	involved in local immune system, and induces cellular growth and differentiation (Ward et al., 2005)
LPT and LPTR	suggested inhibitor of proliferation of MEC (Silva et al., 2002).

* Abbreviations used for the genes: COX1: Cyclooxygenase I; COX2: Cyclooxygenase II; PTGIS: Prostocylcine Synthase; TBXAS: Thromboxane A2 Synthase; CA4: Carbonic Anhydrase IV; ANGPT1: Angiopoietin I; ANGPT2: Angiopoietin II; RTK: Tyrosine Kinase Tie2 Receptor; VEGF: Vascular Endothelial Growth Factor; VEGFR1 (Flt1): Vascular Endothelial Growth Factor Receptor 1 (fms-like tyrosine kinase); VEGFR2 (Flk1): Vascular Endothelial Growth Factor Receptor 2 (Fetal liver kinase 1); BCL2: B-Cell CLL/Lymphoma 2; BAX: Bcl2-Associated X Protein; CCND1: Cyclin D1; IGF1: Insulin-like Growth Factor I; IGF1R: Insulin-like Growth Factor I Receptor; IGFBP3: Insulin-like Growth Factor Binding Protein III; IGFBP5: Insulin-like Growth Factor Binding Protein V; TGFB1: Transforming Growth Factor Beta I; TGFB1R1: Transforming Growth Factor Beta I Receptor I; TGFB1R2: Transforming Growth Factor Beta I Receptor II; PRLR: Prolactin Receptor; LALBA: Alpha-Lactalbumin; LTF: Lactoferrin (Lactotransferrin); LPT: Leptin; LPTR: Leptin Receptor.

Table 2- Accession numbers, amplicon location, range of threshold cycle (CT) values in samples, and slope of standard curve of the analyzed genes

Gene symbol	Accession no.	Gene name	Range of Ct in samples	Standard Curve R^2	Standard curve slope
		Housekeeping genes			
ACTB	AY141970	Beta-Actin	22-32	0.998	-3.53
GAPDH	AF035421	GlycerAldehyde-3-Phosphate Dehydrogenase	28-37	0.999	-3.54
18S RNR1	AY779625	18S Ribosomal RNA I	20-28	0.999	-4.30
		Vascular function & Angiogenesis			
COX1	AF004943	Cyclooxygenase I	30-37	0.998	-3.38
COX2	AF004944	Cyclooxygenase II	33-39	0.917	-3.54
PTGIS	NM_174444	Prostocylcine Synthase	33-39	0.990	-3.62
TBXAS	BC112647	Thromboxane A2 Synthase	31-39	0.999	-3.47
CA4	NM_173897	Carbonic Anhydrase IV	28-39	0.996	-3.19
ANGPT1	NM_001076797	Angiopoietin I	29-38	0.993	-3.50
ANGPT2	AF094699	Angiopoietin II	31-39	0.996	-2.72
RTK	X71424	Tyrosine Kinase Tie2 Receptor	29-38	0.998	-3.72
VEGF	AY114353	Vascular Endothelial Growth Factor	24-35	0.998	-3.53
VEGFR1 (Flt1)	AY114355	Vascular Endothelial Growth Factor Receptor 1 (fms-like tyrosine kinase)	29-38	0.999	-3.23
VEGFR2 (Flk1)	AY114354	Vascular Endothelial Growth Factor Receptor 2 (Fetal liver kinase 1)	29-38	0.999	-3.83
		Cell turnover & Lactogenesis			
BCL2	DQ152929	B-Cell CLL/Lymphoma 2	27-37	0.992	-3.66
BAX	AF163774	Bcl2-Associated X Protein	29-35	0.996	-4.26
CCND1	XM_870776	Cyclin D1	28-39	0.994	-3.66
IGF1	NM_001009774	Insulin-like Growth Factor I	29-38	0.989	-3.70
IGF1R	AF025303	Insulin-like Growth Factor I Receptor	30-38	0.987	-3.71
IGFBP3	M76478	Insulin-like Growth Factor Binding Protein III	26-35	0.994	-3.23
IGFBP5	S52657	Insulin-like Growth Factor Binding Protein V	25-36	0.992	-3.35
TGFB1	M36271	Transforming Growth Factor Beta I	31-39	0.991	-3.77
TGFB1R1	NM_174621	Transforming Growth Factor Beta I Receptor I	29-39	0.976	-3.74
TGFB1R2	NM_615445	Transforming Growth Factor Beta I Receptor II	27-37	0.993	-3.29
PRLR	NM_001039726	Prolactin Receptor	27-36		
LALBA	X63317	Alpha-Lactalbumin	14-32	0.997	-3.81
LTF	L08604	Lactoferrin (Lactotransferrin)	25-39	0.991	-3.70
LPT	OAU84247	Leptin	31-39	0.988	-5.18
LPTR	AY278244	Leptin Receptor	29-39	0.988	-3.71

Table 3- Gene names and primer and probe sequences in real-time RT-PCR

Gene	Forward primer, 5'→3'	Probe, 5'→3'	Reverse primer, 5'→3'
HKG			
ACTB	5'-acccagatcatgttcgagacctt	SYBR GREEN	5'-tcaccggagtccatcacgat
GAPDH	5'-gtcggagtgaacggatttgg	5'-cgcctggtcaccagggctgct	5'-aacgatgtccactttgccagta
18S RNR1	5'- gcaattattccccatgaacg	SYBR GREEN	5'- agttcgaccgtcttctcagc
Vascular Function & Angiogenesis:			
COX1	5'-cctcatcctcatcggggagac	SYBR GREEN	5'-cggttgcggtactggaattgg
COX2	5´-ctaagaagaaagttcattcctgatccc	5'-ttgcccag , LNA#61	5'-gaggatacatctctccattaatcatctg
PTGIS	5'-gccgtcaacagcatcaaacaattc	SYBR GREEN	5'-gcatcagcccaaagccatacc
TBXAS	5'-gcctttggcaccgaagtg	SYBR GREEN	5'-ttatggatggaaatgataagagtaaaacc
CA4	5'-gacatccccagacccaatatgaac	SYBR GREEN	5'-gagcgagcccaggtagcg
ANGPT1	5'-ccataaccagtcagaggcagtac	SYBR GREEN	5'-gtgtgacccttcaaatacaacctg
ANGPT2	5'-cgaatgaagaactcaactacaggattc	5'-ccagccag , LNA#20	5'-gaaggaccacaggcatcaaacc
RTK	5'-aactgtgacgacgaggtgtatg	SYBR GREEN	5'-tccccgcgtaggtgaacttc
VEGF	5'-gggctgctgtaatgacgaaag	SYBR GREEN	5'-tgaggtttgatccgcataatctg
VEGFR1	5'-tcaagccaatgtacaacaggatgg	SYBR GREEN	5'-attaaacttgggagcagaaatatcttcc
VEGFR2	5'-cactgtttatgtgtatgttcaagattac	SYBR GREEN	5'-gatgtacacaacttcatgctggtc
Cell turnover & Lactogenesis:			
BAX	5'-gagtggcggctgaaatgttt	5'-ctggggcc, LNA#57	5'-agggccttgagcaccagttt
BCL2	5'-ggctgggatgcctttgtg	SYBR GREEN	5'-agacagccaggagaaatcaaaca
CCND1	5'-gccgagaagctgtgcatttac	5'-ctccatcc, LNA#58	5'-ccaggaccagctccatgtg
IGF1	5'-tgtgatttcttgaagcaggtgaag	SYBR GREEN	5'-gctgaaggcgagcaagca
IGF1R	5'-tgcagaaggagcaggtgaca	5'-acctgggagccacggcctga	5'-cctccacttgggatccatatttt
IGFBP3	5'-cagagcacagacacccagaa	SYBR GREEN	5'-agggcccgtattctgtctc
IGFBP5	5'-gaccgcaagggattctacaaga	SYBR GREEN	5'-tccacgcaccagcagatg
TGFB1	5'-tggagctgtaccagaaatatagcaa	SYBR GREEN	5'-gccactgccgcacaactc
TGFB1R1	5'-caatgggactagtattctgggaagtag	SYBR GREEN	5'-tcaactgatggatcggaaggta
TGFB1R2	5'-tccttcaagcagacggatgtc	SYBR GREEN	5'-cccgaacggaggctcata
PRLR	5'-catggatactggagtgagtgga	5'-ggatggag, LNA#58	5'-tgtccttcactgggaagtcat
LALBA	5'-acaatggcagcacagaatatgg	SYBR GREEN	5'-tgtcacaggagatgttacagatgttg
LTF	5'-ggatggcaaggaagacttgatc	5'-caggaaga	5'-cgggtggagagccaaagag
LPT	5'- gtgcccatccgcaaggt	5'-caccaggatcaatgac	5'- gacggactgcgtgtgtgaga
LPTR	5'-acccaatgcaatccaatcac	SYBR GREEN	5'-ggctgctcctatgatacctca

Figure legends

Figure 1. The time schedule for sampling relative to drying-off of the gland with a normal lactation (NL) and day of parturition.

CL: continuous lactation BDP, EDP, MDP and LDP: pre-partum sampling times related to the stage in the dry period of the NL gland, i.e. before drying-off, in the early, mid and late dry period, respectively. D: day of lactation. S: stage of gestation-lactation.

Figure 2. Mammary epithelial cells (MEC) were characterized by being poorly differentiated (A), intermediate differentiated (B) or fully differentiated (C), respectively. The tissue samples shown have all been stained with PAS (x400 magnification).

Figure 3. Number of alveoli per picture, and the proportions (%) in mammary sections of alveoli, MEC, lumen, interstitial tissue, micro-vessels, and poorly, intermediate and fully differentiated mammary epithelial cell (MEC).

CL and NL: continuous and normal lactation, respectively. BDP, EDP, MDP and LDP: pre-partum sampling times related to the stage in the dry period of the NL gland, ie. before drying-off, in the early, mid and late dry period, respectively. D: day of lactation.

P values for stage of gestation-lactation (S), CL treatment and their interaction are presented in the top or down right corner of each graph.

Figure 4. Proportion of proliferative (Ki-67) and apoptotic (TUNEL) epithelial cells in mammary sections

CL and NL: continuous and normal lactation, respectively. BDP, EDP, MDP and LDP: pre-partum sampling times related to the stage in the dry period of the NL gland, ie. before drying-off, in the early, mid and late dry period, respectively. D: day of lactation.

P values for stage of gestation-lactation (S), CL treatment and their interaction are presented in the top or down right corner of each graph.

Figure 5. Expression levels of genes related to A) cell turnover and lactogenesis, and B) vascular function and angiogenesis

CL: continuous lactation. NL: normal lactation. BDP, EDP, MDP and LDP: pre-partum sampling times related to the stage in the dry period of the NL gland, ie. before drying-off, in the early, mid and late dry period, respectively. D: day of lactation. Values are least squares means relative to values obtained in the BDP stage of NL glands. *P*-values for stage of gestation-lactation (S), CL treatment and their interaction are presented in the top or down right corner of each graph.

Figure 1.

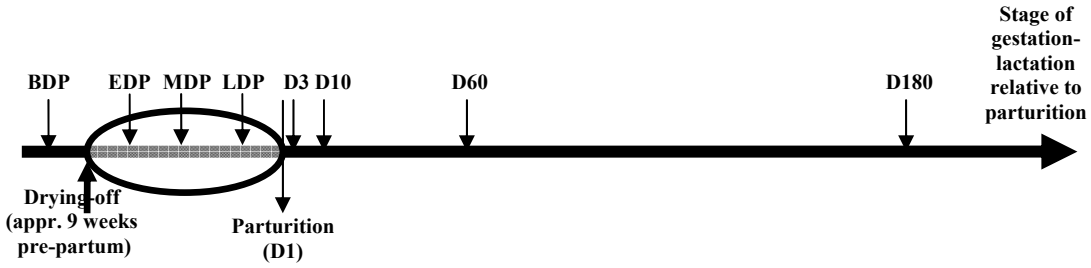

BDP
EDP
MDP
LDP
D3
D10
D60
D180
Stage of gestation-lactation relative to parturition
Drying-off (appr. 9 weeks pre-partum)
Parturition (D1)

Figure 2.

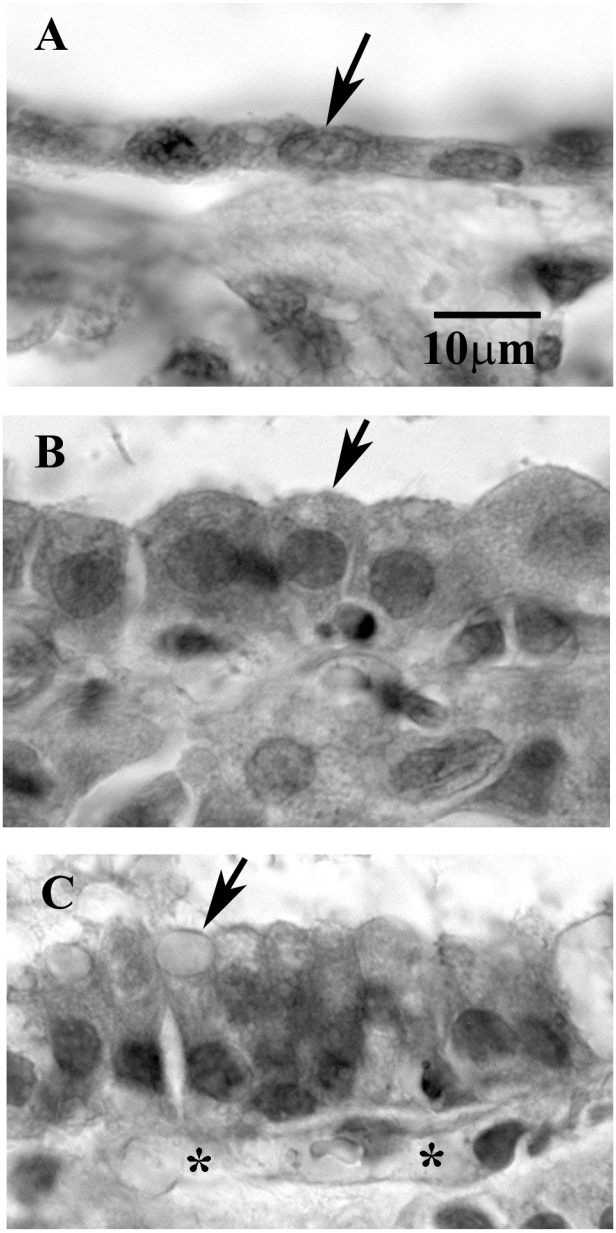


Figure 3.

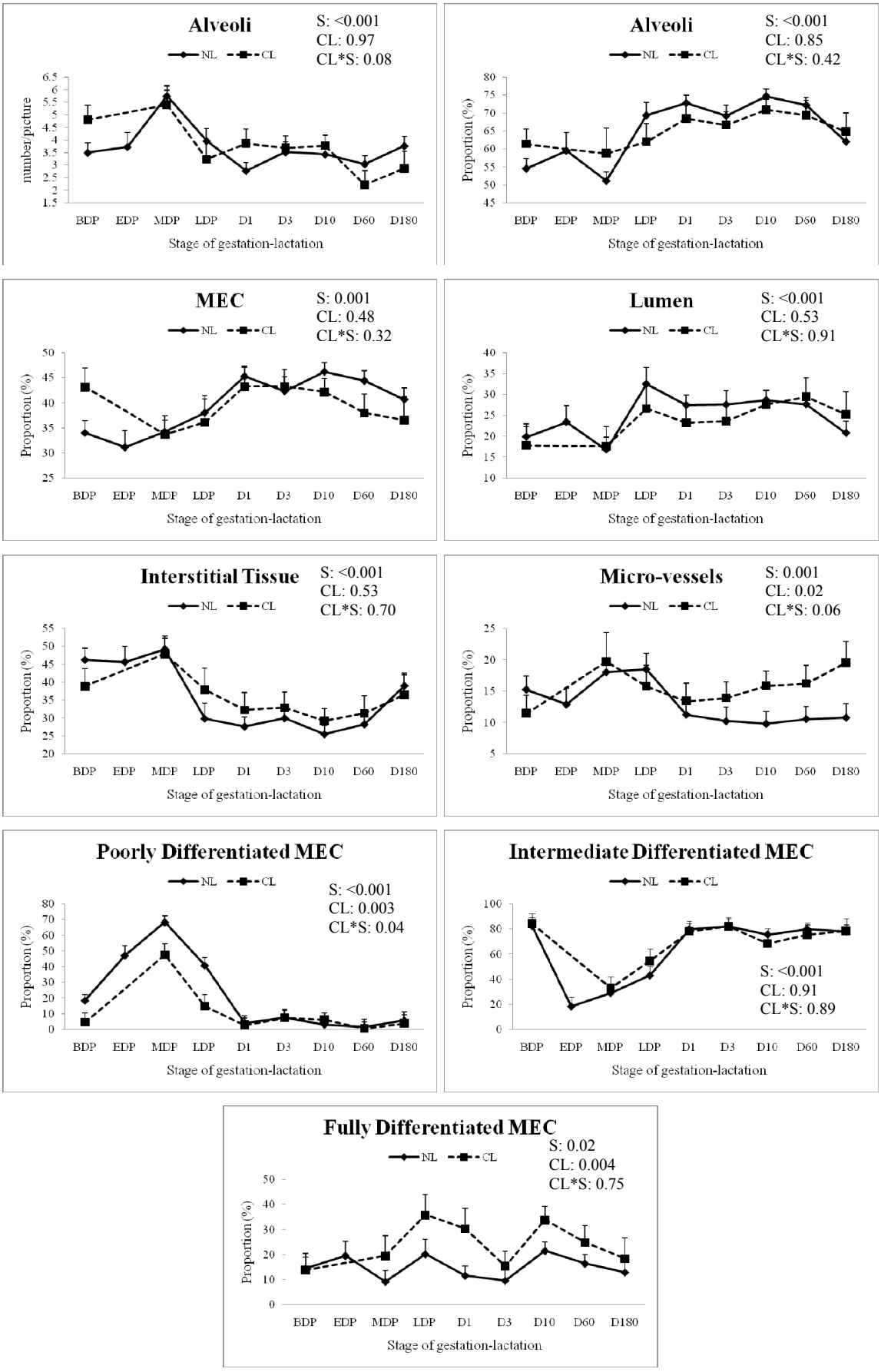

Alveoli
S: <0.001
CL: 0.97
CL*S: 0.08
NL
CL
number/picture
BDP EDP MDP LDP D1 D3 D10 D60 D180
Stage of gestation-lactation
Alveoli
S: <0.001
CL: 0.85
CL*S: 0.42
Proportion (%)
MEC
S: 0.001
CL: 0.48
CL*S: 0.32
Lumen
S: <0.001
CL: 0.53
CL*S: 0.91
Interstitial Tissue
S: <0.001
CL: 0.53
CL*S: 0.70
Micro-vessels
S: 0.001
CL: 0.02
CL*S: 0.06
Poorly Differentiated MEC
S: <0.001
CL: 0.003
CL*S: 0.04
Intermediate Differentiated MEC
S: <0.001
CL: 0.91
CL*S: 0.89
Fully Differentiated MEC
S: 0.02
CL: 0.004
CL*S: 0.75

Figure 4.

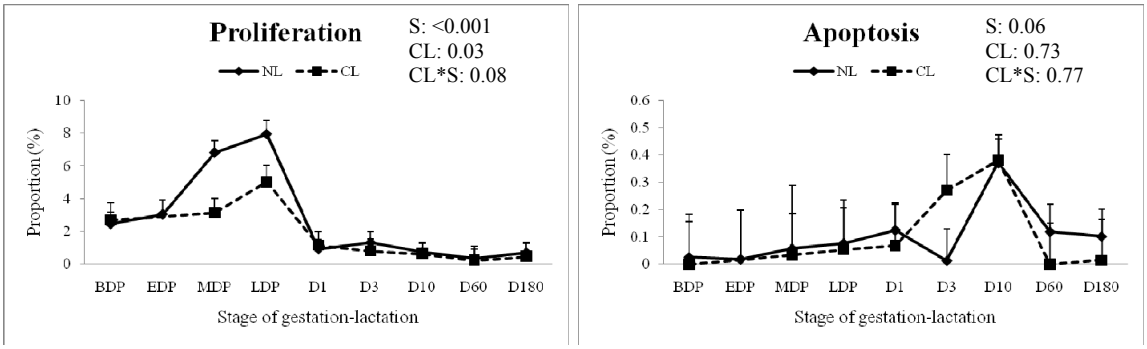
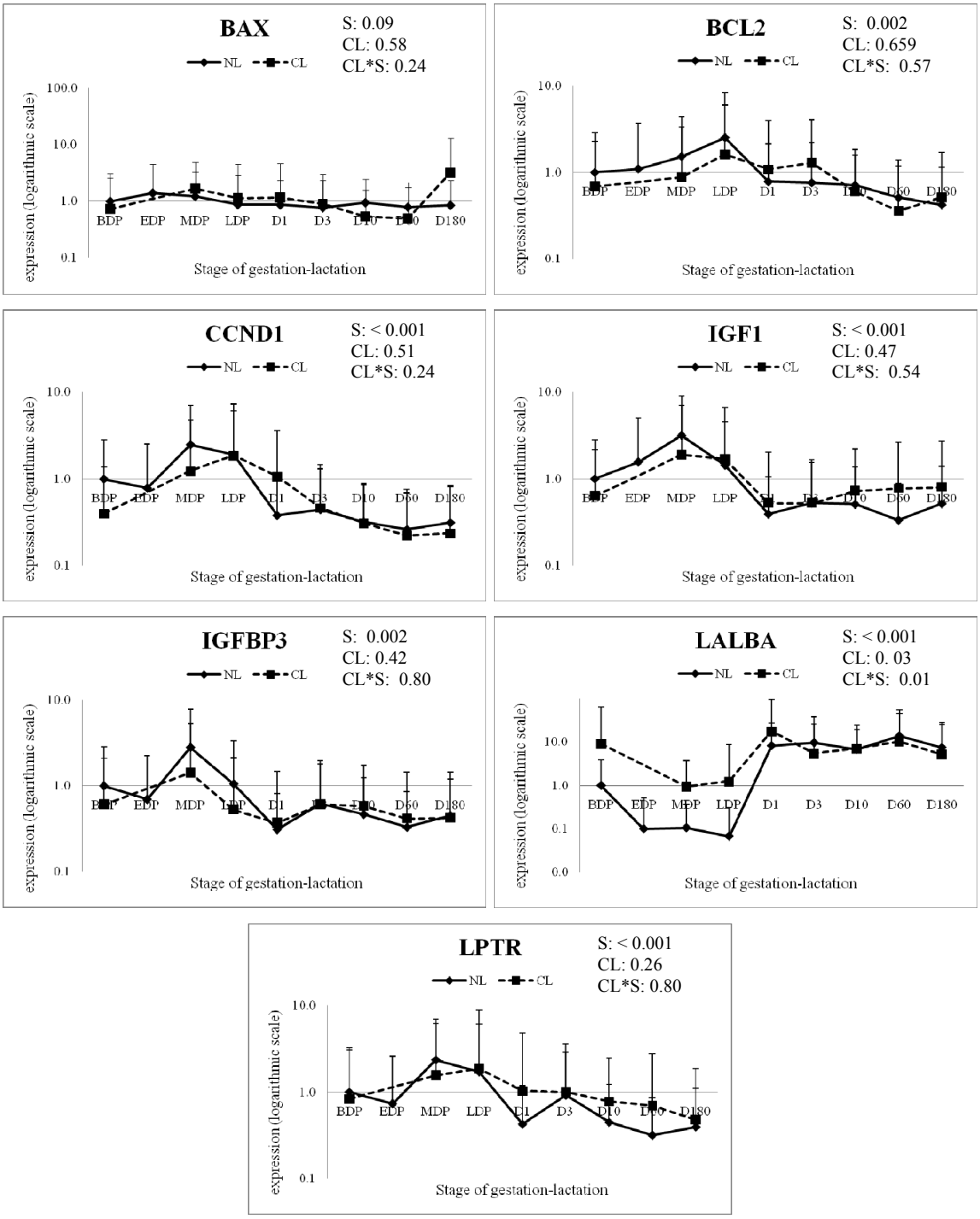
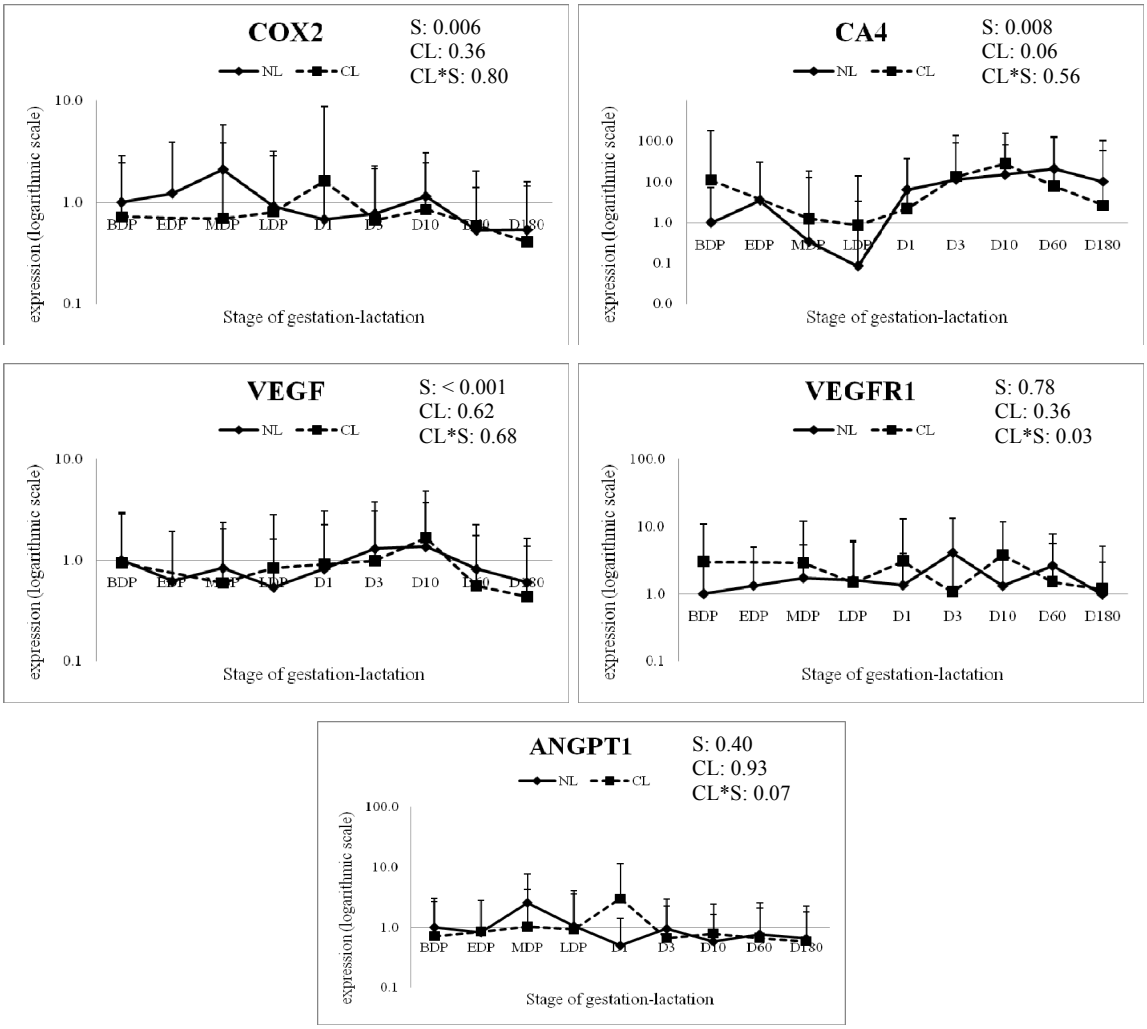

Proliferation
S: <0.001
CL: 0.03
CL*S: 0.08
NL
CL
Proportion (%)
10
8
6
4
2
0
BDP
EDP
MDP
LDP
D1
D3
D10
D60
D180
Stage of gestation-lactation
Apoptosis
S: 0.06
CL: 0.73
CL*S: 0.77
NL
CL
Proportion (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
BDP
EDP
MDP
LDP
D1
D3
D10
D60
D180
Stage of gestation-lactation

Figure 5.

A)


BAX
S: 0.09
CL: 0.58
CL*S: 0.24
NL
CL
expression (logarithmic scale)
100.0
10.0
1.0
0.1
BDP EDP MDP LDP D1 D3 D10 D60 D180
Stage of gestation-lactation
BCL2
S: 0.002
CL: 0.659
CL*S: 0.57
CCND1
S: < 0.001
CL: 0.51
CL*S: 0.24
IGF1
S: < 0.001
CL: 0.47
CL*S: 0.54
IGFBP3
S: 0.002
CL: 0.42
CL*S: 0.80
LALBA
S: < 0.001
CL: 0. 03
CL*S: 0.01
0.0
LPTR
S: < 0.001
CL: 0.26
CL*S: 0.80

B)


COX2
S: 0.006
CL: 0.36
CL*S: 0.80
NL
CL
expression (logarithmic scale)
10.0
1.0
0.1
BDP EDP MDP LDP D1 D3 D10 D60 D180
Stage of gestation-lactation
CA4
S: 0.008
CL: 0.06
CL*S: 0.56
NL
CL
expression (logarithmic scale)
100.0
10.0
1.0
0.1
0.0
BDP EDP MDP LDP D1 D3 D10 D60 D180
Stage of gestation-lactation
VEGF
S: < 0.001
CL: 0.62
CL*S: 0.68
NL
CL
expression (logarithmic scale)
10.0
1.0
0.1
BDP EDP MDP LDP D1 D3 D10 D60 D180
Stage of gestation-lactation
VEGFR1
S: 0.78
CL: 0.36
CL*S: 0.03
NL
CL
expression (logarithmic scale)
100.0
10.0
1.0
0.1
BDP EDP MDP LDP D1 D3 D10 D60 D180
Stage of gestation-lactation
ANGPT1
S: 0.40
CL: 0.93
CL*S: 0.07
NL
CL
expression (logarithmic scale)
100.0
10.0
1.0
0.1
BDP EDP MDP LDP D1 D3 D10 D60 D180
Stage of gestation-lactation

PAPER 2

Continuous Lactation Effects on Mammary Extraction Rates of Nutrients in Dairy Goats

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2009. Pages 652-653 in The XIth International Symposium on Ruminant Physiology (ISRP). Vol. Clermont-Ferrand, France. Y. Chilliard, F. Glasser, Y. Faulconnier, F. Bocquier, I. Veissier, and M. Doreau, eds. Wageningen Academic Publishers, Wageningen, The Netherlands.

INTRODUCTION

Having a dry period appears to be essential for dairy cows to ensure optimal milk production in the following lactation (Madsen et al., 2008). The situation in the dairy goat is less clear, as omission of the dry period (continuous lactation; CL) was reported to have no negative effect on subsequent milk yield in a study by Fowler et al. (1991), while Caja et al. (2006) reported a 29% decrease in milk yield in the subsequent lactation. We have found (unpublished data) that in the goat, mammary glands subjected to CL will enter the new lactation with a smaller mammary epithelial cell (MEC) population, but the MEC are more fully differentiated compared to in the normal lactating (NL) glands. If fully differentiated MEC are older and more secretory active, this could in part explain why goats can lactate continuously without major negative impact on milk yield despite a smaller MEC population. The aim of the present study was to determine if CL in the dairy goat will increase the efficiency of nutrient extraction across the mammary gland in the following lactation, which could reflect differences in MEC secretory activity.

MATERIAL AND METHODS

Nine dairy goats were used followed over one (5 goats) or two consecutive (4 goats) pregnancy-lactation periods. Goats were previously surgically prepared with exteriorized carotid arteries and milk veins. They were milked manually (at 09:00 and 15:30) and fed twice a day (at 07:30 and 14:30), half the ration being given at each feeding. The experimental design was a randomized complete block design; the two udder halves in each animal were randomly assigned to two different treatments, thus using the animal as its own control: One udder half was dried-off approximately 9 weeks pre-partum (NL), and the other udder half was milked continuously (CL) until parturition. After morning and afternoon milking, two blood samples were obtained from the exteriorized carotid artery and each of both milk veins, just before the time of drying-off of the NL gland (BDP), within the first two weeks after drying-off (early dry period; EDP), in the mid-dry period (MDP), within the last two weeks prior to parturition (late dry period; LDP), and at days 1 (day of parturition, D1), 3, 10, 60, and 180 of lactation. Blood samples were taken for immediate determination of acid-base parameters, and plasma was analysed for glucose, non-esterified fatty acids (NEFA), acetate, beta-hydroxy-butyrate (BOHB), triglycerol (TG), and urea. Mammary extraction (E) rates of metabolites were calculated as mammary arterial-milk vein concentration difference

(AVD) divided by arterial concentration. Statistical evaluation of all data was performed using the Proc Mixed procedures in SAS (2003). Variables in the statistical models included the experimental year, treatment (CL or NL), the sampling time (morning or afternoon), stage at which the sample was taken and its interaction with treatment as fixed effects, and the factors goat within experimental year, and milk vein side within goat at each stage as random effects. Pre- and postpartum values were analyzed separately. The PDIFF command in SAS was used to generate comparisons between treatment means. The level of significance was set at $P < 0.05$ and tendencies at $P < 0.10$.

RESULTS

There was not any indication of CL affecting the milk yield negatively in the goats in this study. There was no effect of treatment on acetate AVD and E prepartum, but for glucose and BHB, they were significantly higher in CL than NL glands. Postpartum AVD and E for glucose and BHB were higher compare to prepartum in both glands, but there was no difference between CL and NL glands during the post-partum period. NEFA AVD and E were negative prepartum, and became more negative across CL than NL glands in the LDP ($P=0.01$). Both peaked at the same positive level in both glands at parturition, due to a rise in arterial plasma concentration. They remained high in early lactation, but decreased to become negative again in mid-late lactation, thus following overall changes in arterial plasma concentration. AVD and E for TG were higher across CL than NL glands prepartum, decreased across the gestation period, and then increased after parturition to the same level in both CL and NL glands. The post-partum increase was numerically more marked in NL glands so that the AVD and E reached the same level as in CL glands during lactation. AVD for pO2 and pCO2 as well as AVD and E for TO2 and TCO2 were not affected by the treatment, neither pre- nor postpartum. Prepartum AVD tended to be higher for pH and lower for BE across CL compared with NL glands.

CONCLUSION

AVD and/or E differed for several metabolites either significantly or numerically between the CL and NL glands during the late gestation period. This could reflect differences in extraction activity between the CL glands that remained lactating and the NL glands that were dried off prepartum. However, there was no significant impact of the prepartum milking of the CL

glands on efficiency of mammary nutrient extraction in the subsequent lactation. Our previous findings have shown that the goat mammary gland subjected to CL have lower cell renewal in the subsequent lactation, but MEC on the other hand become more fully differentiated (unpublished data). The observation that extractions of nutrients were unaffected by CL do not lend immediate support to the hypothesis that these more fully differentiated MECs could have a more efficient nutrient extraction reflecting a higher metabolic activity in the subsequent lactation. The explanation why milk yield in dairy goats is relatively unaffected in CL (in contrast to dairy cows) thus remains to be established.

REFERENCES

Caja, G., A. A. K. Salama, and X. Such. 2006. Omitting the dry-off period negatively affects colostrum and milk yield in dairy goats. J. Dairy Sci. 89(11):4220-4228.

Fowler, P. A., C. H. Knight, and M. A. Foster. 1991. Omitting the Dry Period Between Lactations Does Not Reduce Subsequent Milk-Production in Goats. Journal of Dairy Research 58(1):13-19.

Madsen, T. G., M. O. Nielsen, J. B. Andersen, and K. L. Ingvartsen. 2008. Continuous lactation in dairy cows: Effect on milk production and mammary nutrient supply and extraction. J. Dairy Sci. 91(5):1791-1801.

Safayi, S., R. K. Theil, L. Hou, M. Engbaek, J. V. Noergaard, K. Sejrsen, M. O. Nielsen. 2009. Continuous Lactation Effects on Mammary Remodelling During Late Gestation and Lactation in Dairy Goats. Unpublished.

PAPER 3:

Mammary Remodelling in Primiparous and Multiparous Dairy Goats During Lactation

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Accepted by Journal of Dairy Science (JDS-09-2422.R2)

ABSTRACT

Milk production is generally lower, but lactation persistency higher in primiparous (**PP**) than multiparous (**MP**) goats. This may be related to differences in development and maintenance of mammary gland function, but the underlying mechanisms are not well understood. The present study aimed to elucidate whether differences in lactational performance between PP and MP mammary glands are related to the time course of development and maintenance – not only of the mammary epithelial cell (**MEC**) population, but also of the mammary vasculature which sustains synthetic activity. Mammary biopsies were obtained from both mammary glands of 3 PP and 6 MP (≥2 parity) dairy goats at parturition (d1), days 10, 60, and 180 of lactation. Gene transcription relating to MEC turnover and vascular function was quantified by real time RT-PCR, mammary morphology characterised (quantitative histology), and cell turnover determined (TUNEL and Ki-67). Primiparous glands had higher expression for the genes involved in angiogenesis, namely vascular endothelial growth factor receptor 2, and angiopoietin 1 and 2 and their receptor, a few days after parturition (d10). Primiparous glands also had higher rates of MEC cell proliferation in early lactation. It therefore appears that initiation of lactation is associated with development and growth of the mammary gland into early lactation, which continues for a longer period of time in the PP compared to MP glands. In addition, MEC survival was found to be higher in PP glands throughout lactation, and MEC in PP glands underwent more extensive differentiation. This could well explain the reported flatter lactation curve and higher lactation persistency in PP glands. Although some of the genes included in this study were differentially expressed in PP and MP glands during the course of lactation, it was not possible to identify any specific genomic factor(s) that could account for the differences between PP and MP glands with respect to mammary development and MEC survival during lactation. Thus, why parity number impacts MEC and vascular development and survival during lactation, and particularly which regulatory mechanisms are involved, remain to be established.

Key words: primiparous, multiparous, cell turnover, angiogenesis

INTRODUCTION

The level of milk production and the changes in milk yield over the course of lactation, depend on: 1) the number of milk synthesizing epithelial cells, which is affected by the balance between the rate of epithelial cell proliferation and apoptosis (Capuco et al., 2003), and 2) the secretory activity of these cells, which is affected by their differentiation (Akers et al., 2006), and finally 3) the provision of nutrients and removal of waste products via the vascular system essential for sustaining milk synthesis.

Metabolic status through the lactation period in the first lactation (primiparous, **PP**) mammary gland is different from that in the more mature ones (multiparous, **MP**), as the nutrients in PP animals are prioritized not only for lactation but also for the continued growth of the animal (Wathes et al., 2007). Generally, MP animals have a higher milk yield but lower lactation persistency compared to PP animals (Carnicella et al., 2008; Miller et al., 2006). This may be related to differences in development and maintenance of mammary gland function, but the underlying mechanisms are not well understood. We hypothesized that differences between PP and MP goats with respect to milk production and lactation persistency may be related to differences in mammary growth and remodelling also during lactation.

The differential mammary remodelling in PP compared to MP goats may be related not only to the MEC population but also to the vascular system required for nutrient supply and removal of metabolic waste products. Angiogenesis, the formation of new blood vessels, is a biological process which occurs in order to accommodate highly variable requirements for nutrients and oxygenation with respect to changes in mammary tissue composition and metabolic activity (Djonov et al., 2001). This process has received virtually no attention in normal mammary development of dairy animals (Akers, 2002), although the coordinated development of mammary vascular and epithelial cell function must be assumed to be of utmost importance for normal mammary gland function and performance. Vascular function may not be the same in PP and MP glands because the pre-partum development could be different – PP animals not having had a previous lactation with a fully developed mammary gland.

The overall aims of this study were therefore to elucidate whether differences in lactational performance between PP and MP mammary glands are related to the time course of development and maintenance, not only of the MEC population but also of the mammary vasculature which sustains synthetic activity. Furthermore, we aimed to identify the underlying regulatory factors or mechanisms responsible for these developmental differences between PP and MP mammary glands. We aimed to address these issues in a study in dairy

goats and to the extent possible make comparisons to what is known in the dairy cow. Goats have often been used as experimental models for dairy cows in lactation studies due to their low cost and their similar selection for high milk production as dairy cattle (Anderson et al., 1981), but dairy goats appear to differ from dairy cows in their ability e.g. to sustain extended and continuous lactation (omission of the dry period) (Fowler et al., 1991; Mackenzie, 1967). Observations from dairy goats may therefore not be directly applicable to the dairy cow and vice versa. Species comparisons are however fruitful as they can contribute to elucidate general physiological mechanisms underlying mammary function in relation to lactation persistency.

In our study, mammary biopsies were obtained from dairy goats, at parturition and at different time points during lactation. In the biopsies we determined MEC turnover and differentiation, changes in morphology of the micro-vascular system, and the changes in patterns of expression of the major factors responsible for regulation of mammary cell turn-over as well as vascular function and angiogenesis.

MATERIALS AND METHODS

Experimental Animals

Nine Danish crossbred Landrace-Saanen dairy goats were used over two years of experiment: three PP + six MP (parity ≥ 2) goats in the first year, and seven MP goats in the second year (including the three PP + four of the MP goats from the first year). They were fed a diet consisting of hay *ad libitum*, supplemented with barley, concentrate, and molasses, according to their requirements. Animals had free access to fresh water and a vitamin/mineral supplement. They were milked manually at 0900 and 1530 h and fed twice a day at 0730 and 1430 h, half the ration being given at each feeding. The experiment was carried out at the experimental facilities at the Faculty of Life Sciences, University of Copenhagen, Denmark, and approved by the Danish Animal Experimentation Inspectorate, and complied with the Danish Ministry of Justice laws concerning animal experimentation and care for experimental animals.

Experimental Design

Both glands in MP animals were dried off about approximately nine weeks before expected parturition. Two biopsies were sampled from each mammary gland on days 1 (day of parturition), 10, 60, and 180 related to actual kidding. At day 180, both PP and MP animals were in their first month of pregnancy. Biopsying was done according to the procedure

described by Cvek et al. (1998). Before taking biopsies, goats have been milked to be sure there is no milk left in their mammary glands while sampling. To keep the biopsy needle away from major blood vessels near dorsal aspect and midline of the udder and ductal area, the insertion point was marked about 1.5-2 centimeters away from the udder midline, and at the point where the udder protruded the most caudally. To avoid sampling from the same biopsy site and possible scar tissue from each udder half, the biopsy site was measured and noted down at each sampling. One sample was put in RNase-DNase-free tubes and immediately frozen in liquid nitrogen, and stored at -80°C pending analyses. The other biopsy was placed in embedding cassettes and fixed in 4% para-formaldehyde (PFA) solution as described below. Animals were milked both before (to avoid milk in the biopsy) and after (to remove any probable blood clots in the udder) sampling.

Tissue Fixation

Mammary biopsies were fixed in 4% PFA overnight at 4°C, transferred to 70% ethanol for 1-3 days at 4°C, dehydrated using a series of ethanol solutions (70%, 80%, 95% and 100%) (2x60 min each), cleared in xylene (3x60 min), infiltrated with paraffin wax (56-58 ºC) (2x90 min), and finally embedded into paraffin blocks (Histokinet, Shandon Citadel 2000, UK). Blocks were sectioned into 4µm slices (Microtome, Jung, Germany) with at least 30 µm intervals to insure that individual cells were not recounted on successive sections (Ellis and Capuco, 2002). Sections were placed on slides and dried at 37°C overnight for histology purposes and at 60°C for 30 minutes for immunohistochemistry.

Immunohistochemistry

For each sample, 4 sections were put onto each silanated slide (Super Frost Plus, Gerhard Menzel, Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany); one slide was made for Ki-67 antigen localization (**Ki-67**) and another for terminal deoxynucleotidyl transferase dUTP nick end labeling assay (**TUNEL**). Shandon Sequenza Slide Racks (Thermo Electron Corporation, Waltham, MA, USA) and Shandon Coverplates (Thermo Electron Corporation, Waltham, MA, USA) were used for both Ki-67 and TUNEL.

Both Ki-67 and TUNEL have been done according to Nørgaard et. al. (2008b), except for a few modifications:

Ki-67. Before the application of the primary antibody, microwave antigen retrieval protocol was done by boiling sections with 10 mM warm citratbuffer (pH=6.0) in a microwave oven (EM-G4750, SANYO, UK) at 450W (3x5 min).

TUNEL. Slides were digested by proteinase K solution before staining process (Chemicon International Inc., Temecula, CA, USA) (15 min), incubated with equilibration buffer at 4°C for 10 min, and apoptotic cells were stained with diaminobenzidine (DAB) (Sigma-Aldrich Danmark A/S, DK-2605 Brøndby, Denmark) (5 min).

Using the same techniques as for histology, 5 pictures from randomly selected fields of each section at 400x magnifications have been taken and analyzed by ImageJ software, with grid plug-in, to count the number of mammary gland epithelial cells which were positive for proliferation (Ki-67) or apoptosis (TUNEL), as well as total epithelial cell number. A 25% area grid was randomly fitted on pictures and about 700 epithelial cells spread on 20 pictures were counted for each biopsy sample.

Histology

As described by Nøregaard et al. (2008a), paraffin embedded sections were deparaffinized with xylene, rehydrated by serried of alcoholand washed with distilled water. Then, for each of Haematoxylin-Eosin (**H&E**) and Periodic Acid-Schiff (**PAS**) staining, 2-3 sections per sample were used to be processed in the routine lab methods.

All the slides stained with H&E and PAS were studied by light microscopy by the same person, and each sample was assigned a code to mask its identity at the time of analysis to assure unbiased results. From each stained section, three random pictures were taken at primary magnification 400x at a light microscope (Leica DMR, Leica microsystems, Germany) with a camera (Leica DPC 490, Leica microsystems, Germany) connected to computer. Using ImageJ software (Abramoff et al., 2004), a 30 points transparent grid was put on each picture. The number of hits on epithelial cells, lumen, interstitial tissue and micro vessels (in PAS, either their lumen or endothelial cells) were counted. When a point was hitting a mammary epithelial cell (MEC), it was classified into poorly, intermediate or fully differentiated epithelial cell according to its secretory activity as previously described (Akers et al., 2006; Safayi et al., 2009). Furthermore, using an unbiased counting frame with the same size as the picture and having two forbidden lines to decrease the risk of a systematic overestimation, the number of alveoli per picture was counted to fulfill the quantitative measurements. The counting of coinciding points (more than 360 hits per sample) with the mentioned desired structures allowed the unbiased estimation (Gundersen et al., 1988) of volume fractions for each animal.

Real Time Reverse Transcription-PCR

Genes were chosen to be analyzed based on their suggested roles in mammary growth, remodelling and function. Target genes were divided in two groups (see Safayi et al. (2009) for description of physiological function): 1) Genes related to vascular function: cyclooxygenase I (**COX1**), cyclooxygenase II (**COX2**), prostocylcine synthase (**PTGIS**), thromboxane A2 synthase (**TBXAS**) and carbonic anhydrase IV (**CA4**), and angiogenesis: angiopoietin I (**ANGPT1**), angiopoietin II (**ANGPT2**), tyrosine kinase tie2 receptor (**RTK**), vascular endothelial growth factor (**VEGF**), vascular endothelial growth factor receptor 1 (**VEGFR1**) and vascular endothelial growth factor receptor 2 (**VEGFR2**), and 2) Genes related to cell turnover and lactogenesis: B-cell CLL/Lymphoma- 2 (**BCL2**), Bcl2-Associated X Protein (**BAX**), cyclin D1 (**CCND1**), insulin-like growth factor I (**IGF1**), insulin-like growth factor I receptor (**IGF1R**), insulin-like growth factor binding protein III (**IGFBP3**), insulin-like growth factor binding protein V (**IGFBP5**), transforming growth factor beta I (**TGFB1**), transforming growth factor beta I receptor I (**TGFB1R1**), transforming growth factor beta I receptor II (**TGFB1R2**), prolactin receptor (**PRLR**), alpha-lactalbumin (**LALBA**), lactoferrin (**LTF**), leptin (**LPT**) and leptin receptor (**LPTR**). transcription of target genes was quantified by real time RT-PCR. Approximately 10 mg of mammary tissue was homogenized in 350 μL of RNeasy lysis buffer and diluted (1:1) with 70% ethanol. The RNA was purified using the RNeasy mini kit (Qiagen, Albertslund, Denmark) and reverse-transcribed with oligo-dT and Superscript II RNAse H reverse transcription kit (Invitrogen, Taastrup, Denmark) according to the manufacturer's protocol. Reverse-transcribed material (1 μL) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden) using gene-specific primers. Locked nucleic acid (**LNA**) probes from the human Universal ProbeLibrary (Roche Applied Science, Hvidovre, Denmark), labeled with FAM fluorophore were used for detecting amplified LPT, PRL-R, BAX, CCND1, IGF1R, LTF, ANGPT2 and COX2 genes. Power SYBR-Green PCR Master Mix (Applied Biosystems, California, USA) was used for detection of the remaining genes: LPT-R, BCL2, IGF1, IGFBP3, IGFBP5, LALBA, VEGF, VEGF-R1, VEGF-R2, CA4, TBXAS, TGFB1, TGFB1-R1, TGFB1-R2, RTK, ANGPT1, COX1 and PTGIS. Melting curves showed no more than a single amplified product. The PCR amplification signal was detected using an ABI PRISM 7900 detection system (Applied Biosystems). Transcription of glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), 18S ribosomal RNA I (**18S RNR1**) and beta-actin (**ACTB**) were quantified and their appropriateness as house keeping gene (**HKG**) were evaluated against the total RNA content. Transcription of target genes were normalized by the

mean transcription level of GAPDH as HKG, as suggested by Vandesompele et al. (2002) and described by Theil et al. (2006b). The oligonucleotide sequences for the genes were designed using the Primer Express software, version 2.0 (Applied Biosystems Inc, CA, USA) or Beacon Designer Version 7.00 software (Premier Biosoft International, CA, USA). No amplification was found in ribonuclease free water and in samples of genomic bovine DNA. For qRT-PCR, 40 cycles were used at 95°C for 15 s, and 60°C for 60 s. The response was quantified as the number of PCR cycles required to reach a certain threshold, and samples were analyzed in duplicates. The oligonucleotide sequences of forward primers, LNA probes (when used), and reverse primers for the genes are shown in table 2.

Gene expression data were obtained as Ct values (the cycle number at which PCR cycles logarithmic plots cross a calculated threshold line) to evaluate mRNA quantities according to the manufacturer's guidelines, and used to determine ΔCt values:

$$\Delta\text{Ct} = [\text{Ct of the target gene}] - [\text{Ct of the reference gene (GAPDH)}]$$

Least square means of ΔCt values of target genes were normalized to the level observed at d 1 of MP glands, by calculating the $\Delta\Delta$Ct values:

$$\Delta\Delta\text{Ct} = [\Delta\text{Ct observed at a given stage for PP or MP}] - [\Delta\text{Ct observed at d 1 for MP}]$$

The relative mRNA quantity was calculated as $Q^{-\Delta\Delta Ct}$, where Q is 1 + PCR efficiency as determined by $10^{-1/\text{slope of standard curve}}$ -1 (Rasmussen, 2000). In cases with 100% PCR efficiency, this formula was simplified to the following:

$$\text{Relative mRNA quantity (100\% efficiency)} = 2^{-\Delta\Delta\text{Ct}}$$

All statistics were performed at the ΔCt level (Theil et al., 2006b) to exclude potential bias because of averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). Expression of both 18S rRNA and ACTB but not GAPDH were affected by parity. Therefore, GAPDH was selected to be used as housekeeping gene (HKG) for data normalization.

Statistical Analysis

Statistical evaluation of all data was performed using the Mixed procedure in SAS (v.9.1; SAS Institute, USA). Variables in the statistical models included the experimental year, treatment (parity, MP or PP), experiment day at which the biopsy sample has been taken and its interaction with treatment as fixed effects, the factor goat as random effect, and the factor gland within goat at each experiment day was listed in the repeated statement, as shown in the following model:

$Y = \mu + \alpha_i + \beta_j + \gamma_k + \beta_{\gamma jk} + \rho_l + \tau_{klm} + \varepsilon_{ijklm}$, where

Y= the dependent variables, μ= the overall means, α_i= experimental year (i=1,2), β_j= parity (j= MP and PP), γ_k= experiment day (k=1,10,60,180), β_{jk} = interaction between parity and experiment day, ρ_l= random effect of goat (l=1, 2,..,9), τ_{klm} = repeated statement of udder half within a goat at each experiment day (m= left, right), ε_{ijklm}= random variation which was assumed to be normally distributed with a variance σ^2 and a mean of zero. Before performing the final statistical analyses, data were checked for outliers based on residual plots. The data were additionally analyzed in two other ways; 1) including only the goats and glands that were PP in the first year and MP in the second year, and 2) using only PP and MP glands from the first experimental year (excluding year two). The P values differed only slightly compared to the model including the whole set of animals and glands in the two experimental years, and the significancy of the results were the same. Therefore, we decided to use the full model including observations from both years to obtain higher degree of freedom for the MP glands. In addition, contrast analyses were performed at each sampling stage. Furthermore, the data from poorly differentiated MEC as well as cell proliferating and apoptotic cells were arcsin transformed to meet statistical assumptions for normal distribution. However, back-transformed data are presented in tables and graphs. Presented results are expressed as least squares means (LS-Means) with standard error of mean (SEM). The PDIFF option in SAS was used to generate comparisons between treatment means. The level of significance was set at $P < 0.05$.

RESULTS

The general patterns of changes in mammary morphological measures, mammary cell turnover and gene expression were the same in the two experimental years. But levels of alveolar number, proportion of MEC in different differentiation stages and expressions of ANGPT2, VEGF, BCL2, IGFBP3 and TGFB1-R2 differed between the two experimental years, and the reason for this year effect is not known.

Mammary Morphology

In the following, proportions will refer to the number of hits on a given cell or tissue structure compared to the total number of hits, and numbers refer to the total number of that structure observed as explained above.

Gross Morphological Changes. Micro-vessel proportion was neither affected by the stage of lactation nor by parity (results not shown). As shown in figure 1, MP compared to PP glands had higher alveoli number (3.1 versus 2.1) and interstitial tissue proportion (29% and 24%, respectively); but lower alveoli proportion (70% and 75%, respectively) across the whole lactation period. A very high alveoli proportion was observed at parturition (d1) in both MP (72%) and PP (76%) glands. Simultaneously, alveoli number was at its lowest at this time point in both MP (1.7) and PP (2.7) glands, and a very high lumen proportion was observed in PP glands (36%) at parturition, which was significantly higher than observed in the MP ones (26%).

Within the first 10 days after parturition (d10), alveoli number increased in both MP and PP glands (to 3.4 and 2.5, respectively) due to a slight increase in MEC proportion (to 47%), whereas alveoli proportions remained at the same high (MP: 75% and PP: 78%) and interstitial tissue at the same low level (MP: 25% and PP: 21%) as at parturition. Lumen proportion decreased over the first 10 days of lactation in the PP glands to reach the same level as observed in MP glands (27%).

From early into mid-late lactation, a decrease was observed in alveoli proportion (d60: 74% and d180: 65%). MEC proportion remained at the same level from early to mid lactation, but thereafter decreased to the lowest levels in late lactation (42%). From d10 to late lactation lumen proportion remained at the same level (26-27%), but decreased in MP glands to the lowest level (20%) in late lactation. Interstitial tissue proportion started to increase to its peak values, which were observed in late lactation (33%).

Differentiation of MEC. As shown in figure 1, the proportion of poorly differentiated MEC was close to zero in PP glands and significantly lower than in MP glands (3%) during the whole period of lactation. The proportion of intermediate differentiated MEC was numerically ($P = 0.13$) higher in MP compared to PP glands (79% and 69%, respectively); while it was reverse ($P = 0.11$) for the proportion of fully differentiated MEC (16% and 26%, respectively). The proportion of intermediate differentiated MEC proportion was generally high at and a few days after parturition in both glands (79% at d1 and 77% at d10); while, the fully differentiated MEC proportion was low (16% at d1 and 18% at d10). Intermediate differentiated MEC proportion decreased in PP glands in mid and late lactation (60% and 59%, respectively) and became significantly lower than in MP glands, in which proportion of intermediate differentiated MEC remained quite constant in mid late lactation (80%). For fully differentiated MEC proportion the reverse picture was seen, with increases in their proportion in PP glands in mid and late lactation (35% and 34%, respectively), and this

proportion became significantly higher than in MP glands (17% in mid and 13% in late lactation).

Immunohistochemistry

The highest rates of both Ki-67 staining and TUNEL staining were observed at the day of parturition (d1) in PP glands (4.44% and 0.31%, respectively), and those rates were significantly higher ($P = 0.02$ and $P = 0.04$, respectively) than in MP glands (0.84% and 0.08%, respectively). Rates of proliferation and apoptosis were generally quite constant throughout the lactation period in MP glands (approximately 0.55% and 0.07%, respectively), but in PP glands rates of cell proliferation and apoptosis was high in early lactation (2.50% and 0.20%, respectively), and decreased to mid (1.04% and 0.06%, respectively) and late (0.80% and 0.01%, respectively) lactation stages. But as shown in figure 2, rates of cell proliferation were consistently higher in PP glands throughout the lactation period.

Gene Expression

Genes Related to Cell Turnover and Lactogenesis. IGF1 ($P = 0.03$), LPT ($P = 0.004$), LPTR ($P = 0.04$) were more, and BCL2 ($P = 0.07$) and IGFBP3 ($P = 0.07$) tended to be less expressed in PP compared to MP glands. As shown in figures 3A and 3B, BCL2, IGF1, IGFBP3, IGFBP5, TGFB1, TGFB1R2, PRLR, LALBA, LTF, LPT and LPTR were at a low or their lowest expression level at parturition time, and for BCL2 ($P = 0.04$), TGFB1 ($P < 0.01$), PRLR ($P = 0.01$) and LALBA ($P = 0.01$) lower in PP than MP glands. BCL2 and IGFBP3 at d10 ($P < 0.001$ and $P = 0.03$, respectively) were lower expressed in PP than MP, but reached the same level in both glands at mid-late lactation. TGFB1, TGFB1R2, PRLR, LTF, LALBA and LPTR increased in PP glands at d10, made TGFB1R2 ($P = 0.01$) and PRLR ($P = 0.01$) higher in those than in MP glands. TGFB1 and LALBA at d10 and TGFB1R2 and PRLR at d60 reached the same level in both glands till late lactation. IGFBP5 peaked in both glands in mid lactation. LTF was the same level in both glands, increased to peak in late lactation. IGF1, LPT and LPTR remained constant in MP. LPT and LPTR increased in PP glands, in a way similar to IGF1 and LPTR in mid lactation ($P < 0.05$ and $P = 0.04$, respectively) and IGF1, LPT and LPTR in late lactation were higher in PP than in MP glands ($P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively). There was neither effect of stage of lactation nor treatment on the expression of BAX, CCND1, IGF1R and TGFB1R1 (data not shown).

Genes Related to Vascular Function and Angiogenesis. As shown in figure 3B, in PP compared to MP glands, COX2 and PTGIS were higher and VEGF tended to be less ($P < 0.10$) expressed. The expression of all other studied genes in this group were constant in MP glands, except for peaked expression of VEGF at d10 and for VEGFR1, ANGPT1 and RTK in mid lactation.

The case in PP glands was different from that in MP glands as their expression at parturition was either low or at its lowest level, in a way that for TBXAS ($P = 0.02$), VEGFR1 ($P = 0.01$) and RTK ($P = 0.04$), was significantly lower than in MP glands. Expression of VEGF, VEGFR2, ANGPT1 and ANGPT2 was raised or peaked at d10, and became higher in PP than in MP ($P = 0.03$, $P = 0.03$, $P < 0.01$ and $P = 0.03$, respectively). From D10 onwards, expression of VEGF, VEGFR1, VEGFR2 and ANGPT2 and RTK remained at the same high level. COX1, COX2, PTGIS, TBXAS increased to peak in late lactation, made them higher in PP than in MP glands ($P < 0.01$, $P < 0.01$, $P < 0.01$ and $P = 0.09$, respectively). There was neither any effect of stage of lactation nor the treatment on the expression of CA4, although it was numerically lower in PP compared to MP glands.

DISCUSSION

It has been shown in dairy goats (Carnicella et al., 2008; Mioc et al., 2008) as well as dairy cows (Miller et al., 2006), that PP animals have a lower but more persistent milk production compared to MP animals. Unfortunately, the milk yield data in this experiment for the first 19 weeks of lactation were lost, but in agreement with these previous reports, PP goats produced about 20% less milk than MP goats during lactation weeks 20 to 30 of lactation where the last samples were taken (results not shown). The difference became numerically smaller by the end of this period, but the data did not allow us to evaluate lactation persistency statistically.

Impact of Parity Number on MEC Development During Lactation

We observed a very low rate of cell proliferation and hardly any visible changes in this rate over the course of lactation in MP glands, in which renewal of cells mainly takes place prior to parturition (Caja et al., 2006). In contrast, cell renewal in PP glands was substantial after parturition. This continued growth in PP mammary gland has been previously reported in dairy goats (Anderson et al., 1981; Knight and Peaker, 1984) as well as in dairy cows (Miller et al., 2006). Furthermore, rates of apoptosis were high in both MP and PP glands right after parturition, but decreased to almost zero in PP glands over the course of lactation. The MEC:lumen ratio was substantially lower in PP (1.0:1) compared to MP glands (1.6:1) at the

time of parturition, but by d 60 of lactation the ratio in PP glands (1.8:1) had increased to the same level as in MP glands (1.7:1). This indicates that PP glands are less compositionally differentiated at parturition compared to MP glands. Thus it seems that MEC proliferation as well as differentiation continues further into lactation in PP compared to MP goats.

There was a very low proportion of poorly and a high proportion of intermediately and fully differentiated MEC in both PP and MP glands during established lactation, which has also been previously reported in Holstein cows (Akers et al., 2006). In our study, the proportion of intermediately differentiated MEC decreased over the course of lactation in PP glands, whilst the proportion of fully differentiated MEC increased, especially from d10 and onwards. These changes most likely reflect that intermediately differentiated MEC progressively become differentiated into fully differentiated MEC in PP glands. As a consequence of lower rate of apoptosis from d10 and onwards in PP compared to MP glands, this would overall indicate that the MEC population in PP glands in addition to having new cells added during lactation, also were more persistent and hence capable of remaining lactating for a longer period of time.

Impact of Parity Number on Key Regulatory Factors in MEC Turnover

Parity significantly impacted expression of the majority of genes involved in regulation of cell turnover and lactogenesis (BCL2, IGF1, IGFBP3, IGFBP5, TGFB1, TGFB1R2, PRLR, LALBA, LPT and LPTR), but not BAX, CCND1, IGF1R, TGFB1R1 and LTF. In recent studies in MP cows (Norgaard et al., 2005), sheep (Norgaard et al., 2008a) and goats (Caja et al., 2006), it was found that the major part of mammary redevelopment occurs in late pregnancy. It was therefore not surprising that the level of expression of many of the genes involved in cell turnover was low at the time of parturition in our dairy goats, when lactation had been initiated.

Expression of the antiapoptotic factor BCL2 (Reed, 1998) corresponded with high rates of apoptosis at parturition and higher apoptotic rates in PP compared to MP glands. We were unable to relate differences in rates of apoptosis during the lactation period or between PP and MP glands to expression of BAX, which is an apoptotic factor (Reed, 1998). Although this suggests that the antiapoptotic factor BCL2 plays a greater role than BAX in regulation of apoptosis from parturition and during lactation, it can not be ruled out that BAX may be involved in this regulation as well. The present as well as previous gene expression studies have been conducted on biopsies of mammary tissue consisting of a variety of different tissues and cell types present in the mammary gland. Gene expression changes occurring in a

particular cell type may therefore be masked by the presence of other tissue structures. There is a need in the future to conduct tissues specific expression studies, using new techniques like laser microdissection to separate individual cell types in order to shed more light on the coordinated regulation of mammary development and remodeling.

Initiation of lactation in MP ruminants is associated with up-regulation of the PRLR in MEC and initiation of copious milk secretion including the whey protein LALBA, and in this study expression of these factors was at therefore at its highest at the time of parturition in MP goats. However, in PP goats both PRLR and LALBA expression increased from parturition to peak at d10, which is yet another indication that initiation of lactation and growth in the PP mammary gland continues further into early lactation and that initiation of lactation possibly is delayed or a prolonged process in PP glands of dairy goats. Prolactin and its receptor are involved in > 300 biological processes (Bole-Feysot et al., 1998). PRLR is a determinant for prolactin signaling, and stimulates cellular proliferation, differentiation and lactogenesis in MEC (Wall et al., 2006), and PRL/PRLR has been demonstrated to be important for milk removal and mammary function in dairy cows (Wall et al., 2006). Moreover, Theil et al. (2006a) showed that expression of PRLR in mammary glands of sows is crucial in order to ensure continued growth of the mammary gland at parturition and to onset lactation. The PRLR could therefore be one of the overall factors involved in coordinating mammary growth, function and angiogenesis in the periparturient dairy goat, and the delayed lactogenesis and continued development of the PP mammary gland into lactation could be a result of sustained expression of the PRLR. The underlying reason for this differential expression pattern in PP and MP goats' mammary glands is not known.

Insulin-like growth factor 1 has been reported to stimulate MEC growth, proliferation and cell survival (Knight, 2000) and LPT has been reported to be involved in regulation of MEC proliferation and/or differentiation prior to parturition (Chilliard et al., 2001). We found that expression of IGF1, LPT and LPTR were at similar levels in the MP and PP mammary gland at the time of parturition, but with progressing lactation, expression of these factors increased in PP glands, whereas expression levels remained constant or even diminished in the MP mammary glands. This coincided with the more pronounced shift from poorly over intermediately to fully differentiated MEC in PP compared to MP glands. It is therefore tempting to speculate that IGF-1 and LPT are important factors involved in regulating differentiation and cell survival during lactation in general, and also contributing to explain the different developmental patterns between PP and MP glands over the course of lactation.

The regulatory factors determining development and differentiation of MEC during the normal lactation in MP goats thus seem to be the same set of factors responsible for the differential pattern of development in the PP compared to the MP mammary gland. It would be valuable to gain insight into the underlying reason for the impact of parity on expression of this whole range of MEC genes, as this is a key to understanding aspects of lactation persistency.

Impact of Parity Number on Vascular Development During Lactation and Key Regulatory Mechanisms

In contrast to most other organs, where the angiogenic potential is only used in response to injury, the mammary parenchyma is persistently regulating its vasculature (Djonov et al., 2001). There are a few studies available on the mammary vasculature in the normal mammary gland during the course of gestation and lactation (Akers, 2002), but to the best of our knowledge, no previous studies have looked into the regulatory mechanisms responsible for vascular remodeling and function during the normal course of lactation in ruminants.

In the present study, the proportion of micro-vessels in mammary tissue was neither affected by stage of lactation nor by parity. We performed an additional statistical analysis on our data to calculate the micro-vessel to interstitial tissue ratio in the interstitial tissue proportion only, i.e. the proportion of micro-vessels within the interstitial tissue rather than in the whole mammary tissue including MEC. The micro-vessel to interstitial tissue ratio was markedly higher ($P < 0.0001$) in PP (71%) than MP (41%) glands during the whole lactation period. This ratio decreased in parallel in PP and MP glands from early lactation and reached the lowest levels in late lactation in both PP and MP glands (59% and 31%, respectively). These overall lactation changes are equivalent to what has been previously reported in rats (Yasugi et al., 1989). The pattern of changes in the above ratio over the course of lactation reflects microvasculature regression. In the mouse, this has been shown to follow the alveolar degeneration (Matsumoto et al., 1992). Based on another study on vascular remodeling in the mouse mammary gland, Djonov et al. (2001) suggested that regression of the endothelium could be correlated to, or even be a consequence of, MEC involution. Whatever comes first, vascular or alveolar degeneration, it appears evident that the function and integrity of the MEC is dependent on and closely coordinated with the vascular system responsible for provision of nutrients and removal of waste products, essential to sustain MEC metabolism.

This is the first report of quantitative gene expression analysis for factors involved in angiogenesis and vascular remodeling (VEGF, VEGFR1, VEGFR2, ANGPT1, ANGPT2 and

RTK) in the mammary gland in dairy goats during lactation. We are not aware of any such studies in the bovine, and only few studies in rodents have looked into the mammary vasculature and regulation of its remodeling (Djonov et al., 2001; Pepper et al., 2000). Parity appeared to influence the whole range of studied genes associated with vascular function and angiogenesis in our studied dairy goats. VEGFR1, VEGFR2, ANGPT1, ANGPT2 and RTK are all reported to be angiogenic factors (Shibuya, 2001; Thurston, 2003), and these genes as well as PRLR and LALBA were generally higher expressed in MP compared to PP at the time of parturition, but this had reversed by d10, where highest expression levels were found in PP glands. It has been suggested that the MEC is the main source of angiogenic factors in the mammary gland (Pepper et al., 2000), and as such it appears logical that expression of angiogenic factors would follow the developmental pattern of MEC described above during lactation and in mammary glands of different parity. Primiparous glands thus continue to develop also their vascular system post-partum to a greater extent than in MP goats. Furthermore, we observed that a number of other genes (COX1, COX2, PTGIS and TBXAS) encoding for factors regulating vascular tone and function (Nielsen et al., 1995) were higher expressed in PP than MP glands, especially in late lactation. These factors are synthesized within the capillary endothelial cells in addition to MEC. Cyclooxygenase (COX1 and COX2) are key enzymes involved in synthesis of a number of vaso-active substances (Simmons et al., 2004), and the enzyme PTGIS specifically regulates the synthesis of prostacyclin (PGI2), which has been reported to be a potent vasodilator in the mammary gland (Nielsen et al., 2004). Higher expression of genes encoding for potent vasodilators could likely be associated with improved blood perfusion in PP glands in more advanced stages of lactation, and would thus contribute to explain why lactation persistency may be higher in PP goats. The reason for higher expression of these genes encoding for factors with vasoactive action is not known. It is noteworthy that IGF1, LPT and LPTR exhibited the same pattern of expression changes as the vasoactive factors COX1, COX2, PTGIS and TBXAS. This could suggest that the differential expression of the vascular function genes was linked to the differential development of the MEC in PP compared to MP glands, and in this way coupled to differential development also of the mammary vascular system. An important message is that the changes in vascular and MEC function appear to be regulated in a coordinated manner. Our study gives rise to the question whether the vascular system may be involved in determining lactation persistency, and this issue deserves to receive more attention in the future, as so little work has been carried out in this area so far.

CONCLUSION

We have found that initiation of lactation and growth of the mammary gland continues further into early lactation in the PP compared to the MP dairy goats. This not only applies to MEC renewal, but also to development of vascular function (angiogenesis). This combined with improved MEC survival and maintenance of vascular competence, could explain the reported higher lactation persistency in PP compared to MP goats. There is an orchestrated change taking place to regulate the MEC and vascular function during normal lactation and to ensure coordination of function in these two important tissue components of the mammary gland. We have provided evidence that the set of genes regulating these orchestrated changes, are also basically the ones which can explain the different patterns of mammary development and performance during lactation between PP and MP goats, and hence the factors encoded for by these genes are likely to be important determinants of lactation persistency. We therefore suggest that development and function of the microvasculature may be just as essential for the overall integrity of the mammary gland and lactation persistency as the MEC. This issue should be addressed in future studies. The underlying reasons for the differential expression in PP compared to MP goats of genes responsible for regulation of MEC and vascular development remain to be established.

ACKNOWLEDGMENTS

This project was financed by the Danish Research Council for Technology and Production Sciences. Sina Safayi was in receipt of a PhD scholarship co-financed by the Danish Research Council and by Mrs. M. Namian. The authors would like to acknowledge V.G. Christensen, D.S. Jensen, R. Jensen, K.B. Poulsen. and H.A.V. Ruby for their valuable technical assistance. We also wish to express our gratitude to Dr. C.Th. Ekstrøm for his advices in statistics and Dr. Dvora-Laiô Wulfsohn for hers in stereology.

REFERENCES

Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image Processing with ImageJ. Biophotonics International 11(7):36-42.

Akers, R. M. 2002. Lactation and the Mammary Gland. First ed. Blackwell publishing, Ames, Iowa, USA.

Akers, R. M., A. V. Capuco, and J. E. Keys. 2006. Mammary histology and alveolar cell differentiation during late gestation and early lactation in mammary tissue of beef and dairy heifers. Livestock Science 105(1-3):44-49.

Anderson, R. R., J. R. Harness, A. F. Snead, and M. S. Salah. 1981. Mammary Growth Pattern in Goats during Pregnancy and Lactation. J. Dairy Sci. 64(3):427-432.

Bole-Feysot, C., V. Goffin, M. Edery, N. Binart, and P. A. Kelly. 1998. Prolactin (PRL) and its receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocrine Reviews 19(3):225-268.

Caja, G., A. A. K. Salama, and X. Such. 2006. Omitting the dry-off period negatively affects colostrum and milk yield in dairy goats. J. Dairy Sci. 89(11):4220-4228.

Capuco, A. V., S. E. Ellis, S. A. Hale, E. Long, R. A. Erdman, X. Zhao, and M. J. Paape. 2003. Lactation persistency: Insights from mammary cell proliferation studies. Journal of Animal Science 81:18-31.

Carnicella, D., M. Dario, M. C. C. Ayres, V. Laudadio, and C. Dario. 2008. The effect of diet, parity, year and number of kids on milk yield and milk composition in Maltese goat. Small Ruminant Research 77(1):71-74.

Chilliard, Y., M. Bonnet, C. Delavaud, Y. Faulconnier, C. Leroux, J. Djiane, and F. Bocquier. 2001. Leptin in ruminants. Gene expression in adipose tissue and mammary gland, and regulation of plasma concentration. Domestic Animal Endocrinology 21(4):271-295.

Djonov, V., A. C. Andres, and A. Ziemiecki. 2001. Vascular remodelling during the normal and malignant life cycle of the mammary gland. Microscopy Research and Technique 52(2):182-189.

Ellis, S., and A. V. Capuco. 2002. Cell proliferation in bovine mammary epithelium: identification of the primary proliferative cell population. Tissue & Cell 34(3):155-163.

Fowler, P. A., C. H. Knight, and M. A. Foster. 1991. Omitting the Dry Period Between Lactations Does Not Reduce Subsequent Milk-Production in Goats. Journal of Dairy Research 58(1):13-19.

Gundersen, H. J. G., T. F. Bendtsen, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J. R. Nyengaard, B. Pakkenberg, F. B. Sorensen, A. Vesterby, and M. J. West. 1988. Some New, Simple and Efficient Stereological Methods and Their Use in Pathological Research and Diagnosis - Review Article. Apmis 96(5):379-394.

Knight, C. H. 2000. The importance of cell division in udder development and lactation. Livestock Production Science 66(2):169-176.

Knight, C. H., and M. Peaker. 1984. Mammary Development and Regression During Lactation in Goats in Relation to Milk Secretion. Quarterly Journal of Experimental Physiology and Cognate Medical Sciences 69(2):331-338.

Mackenzie, D. 1967. Goat Husbandry. 2nd ed. Faber & Faber, London.

Matsumoto, M., H. Nishinakagawa, M. Kurohmaru, Y. Hayashi, and J. Otsuka. 1992. Pregnancy and Lactation Affect the Microvasculature of the Mammary-Gland in Mice. Journal of Veterinary Medical Science 54(5):937-943.

Miller, N., L. Delbecchi, D. Petitclerc, G. F. Wagner, B. G. Talbot, and P. Lacasse. 2006. Effect of stage of lactation and parity on mammary gland cell renewal. J. Dairy Sci. 89(12):4669-4677.

Mioc, B., Z. Prpic, I. Vnucec, Z. Barac, V. Susic, D. Samarzija, and V. Pavic. 2008. Factors affecting goat milk yield and composition. Mljekarstvo 58(4):305-313.

Nielsen, M. O., I. R. Fleet, K. Jakobsen, and R. B. Heap. 1995. The Local Differential Effect of Prostacyclin, Prostaglandin E(2) and Prostaglandin-F2-Alpha on Mammary Blood-Flow of Lactating Goats. Journal of Endocrinology 145(3):585-591.

Nielsen, M. O., S. Nyborg, K. Jakobsen, I. R. Fleet, and J. Norgaard. 2004. Mammary uptake and excretion of prostanoids in relation to mammary blood flow and milk yield during pregnancy-lactation and somatotropin treatment in dairy goats. Domestic Animal Endocrinology 27(4):345-362.

Norgaard, J., A. Sorensen, M. T. Sorensen, J. B. Andersen, and K. Sejrsen. 2005. Mammary cell turnover and enzyme activity in dairy cows: Effects of milking frequency and diet energy density. J. Dairy Sci. 88(3):975-982.

Norgaard, J. V., M. O. Nielsen, P. K. Theil, M. T. Sorensen, S. Safayi, and K. Sejrsen. 2008a. Development of mammary glands of fat sheep submitted to restricted feeding during late pregnancy. Small Ruminant Research 76(3):155-165.

Norgaard, J. V., M. T. Sorensen, P. K. Theil, I. Sehested, and K. Sejrsen. 2008b. Effect of pregnancy and feeding level on cell turnover and expression of related genes in the mammary tissue of lactating dairy cows. Animal 2(4):588-594.

Pepper, M. S., D. Baetens, S. J. Mandriota, C. Di Sanza, S. Oikemus, T. F. Lane, J. V. Soriano, and R. Montesano. 2000. Regulation of VEGF and VEGF receptor expression in the rodent mammary gland during pregnancy, lactation, and involution. Developmental Dynamics 218(3):507-524.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29(9).

Rasmussen, R. P. 2000. Quantification on the LightCycler. Rapid Cycle Real-Time PCR, Methods and Applications (eds S Meuer, CT Wittwer and K Nakagawara):21-34.

Reed, J. C. 1998. Bcl-2 family proteins. Oncogene 17(25):3225-3236.

Safayi, S., P. K. Theil, L. Hou, M. Engbaek, J. V. Norgaard, K. Sejrsen, and M. O. Nielsen. 2009. Continuous lactation effects on mammary remodelling during late gestation and lactation in dairy goats. J. Dairy Sci. doi:10.3168/jds.2009-2507.

Shibuya, M. 2001. Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. Cell Structure and Function 26(1):25-35.

Simmons, D. L., R. M. Botting, and T. Hla. 2004. Cyclooxygenase isozymes: The biology of prostaglandin synthesis and inhibition. Pharmacological Reviews 56(3):387-437.

Theil, P. K., K. Sejrsen, W. L. Hurley, R. Labouriau, B. Thomsen, and M. T. Sorensen. 2006a. Role of suckling in regulating cell turnover and onset and maintenance of lactation in individual mammary glands of sows. Journal of Animal Science 84(7):1691-1698.

Theil, P. K., I. L. Sorensen, M. Therkildsen, and N. Oksbjerg. 2006b. Changes in proteolytic enzyme mRNAs relevant for meat quality during myogenesis of primary porcine satellite cells. Meat Science 73(2):335-343.

Thurston, G. 2003. Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. Cell and Tissue Research 314(1):61-68.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3(7):research0034.

Wall, E. H., H. M. Crawford, S. E. Ellis, G. E. Dahl, and T. B. McFadden. 2006. Mammary response to exogenous prolactin or frequent milking during early lactation in dairy cows. J. Dairy Sci. 89(12):4640-4648.

Wathes, D. C., Z. Cheng, N. Bourne, V. J. Taylor, M. P. Coffey, and S. Brotherstone. 2007. Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. Domestic Animal Endocrinology 33(2):203-225.

Yasugi, T., T. Kaido, and Y. Uehara. 1989. Changes in Density and Architecture of Microvessels of the Rat Mammary-Gland During Pregnancy and Lactation. Archives of Histology and Cytology 52(2):115-122.

Figure legends

Figure 1. Number of alveoli per picture, and the proportions (%) in mammary sections of alveoli, MEC, lumen, interstitial tissue, micro-vessels, and poorly, intermediate and fully differentiated MEC.

PP and MP: primiparous (broken line) and multiparous (full line) glands, respectively. D: day of lactation. P values for stage of gestation-lactation (S), Parity (P) and their interaction are presented in the top or down right corner of each graph.

Figure 2. Percentile of proliferative (Ki-67) and apoptotic (TUNEL) epithelial cells in mammary sections.

PP and MP: primiparous (broken line) and multiparous (full line) glands, respectively. D: day of lactation. P values for stage of gestation-lactation (S), Parity (P) and their interaction are presented in the top or down right corner of each graph.

Figure 3. Expression levels of genes related to A) cell turnover and lactogenesis, and B) vascular function and angiogenesis

PP and MP: primiparous (broken line) and multiparous (full line) glands, respectively. D: day of lactation. Values are relative to d1 of MP glands and LSMeans, and 95% confidence limits. P values for stage of gestation-lactation (S), Parity (P) and their interaction are presented in the top or down right corner of each graph.

Figure 1.

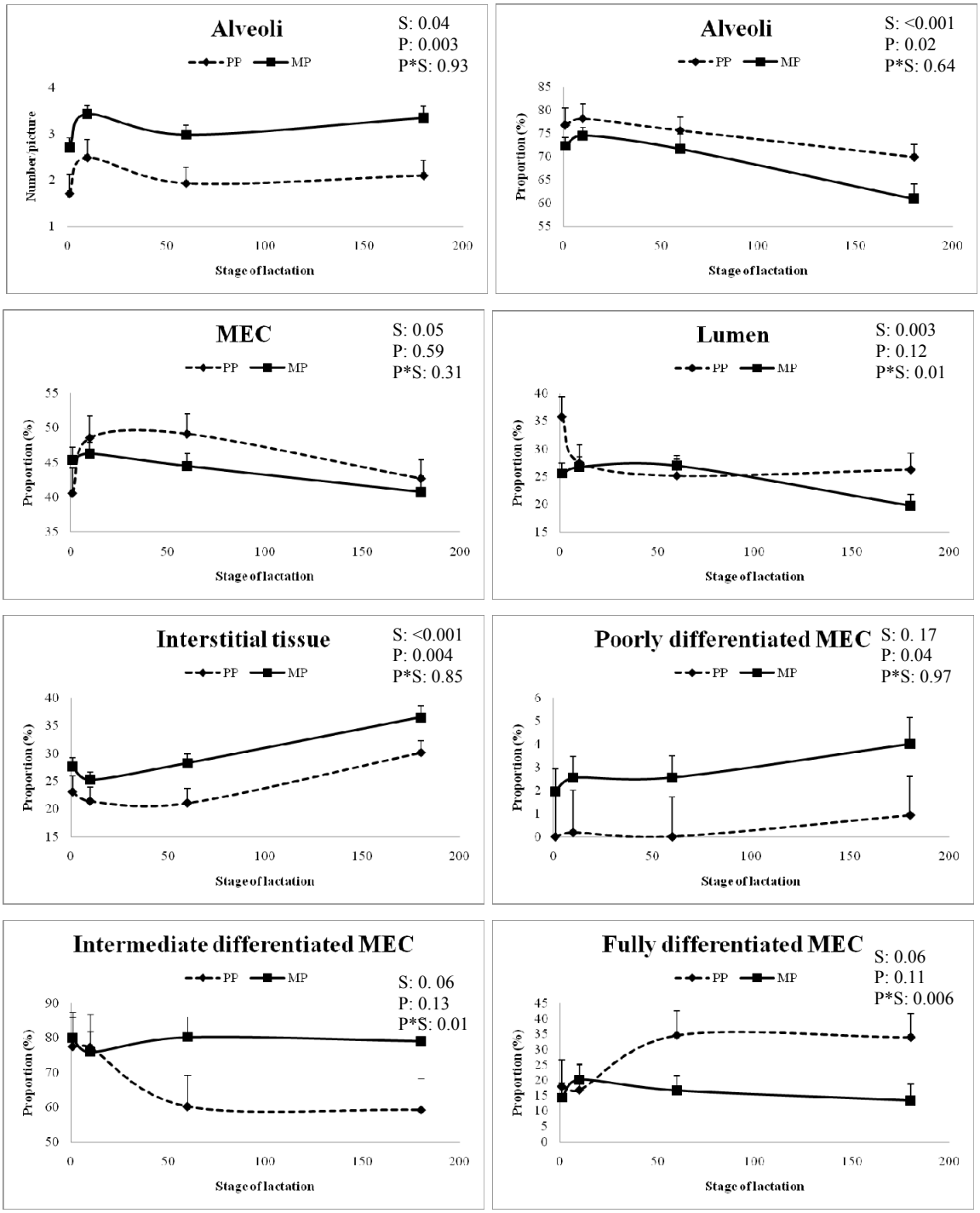

Alveoli
S: 0.04
P: 0.003
P*S: 0.93
PP
MP
Number picture
Stage of lactation
Alveoli
S: <0.001
P: 0.02
P*S: 0.64
Proportion (%)
MEC
S: 0.05
P: 0.59
P*S: 0.31
Lumen
S: 0.003
P: 0.12
P*S: 0.01
Interstitial tissue
S: <0.001
P: 0.004
P*S: 0.85
Poorly differentiated MEC
S: 0. 17
P: 0.04
P*S: 0.97
Intermediate differentiated MEC
S: 0. 06
P: 0.13
P*S: 0.01
Fully differentiated MEC
S: 0.06
P: 0.11
P*S: 0.006

Figure 2.

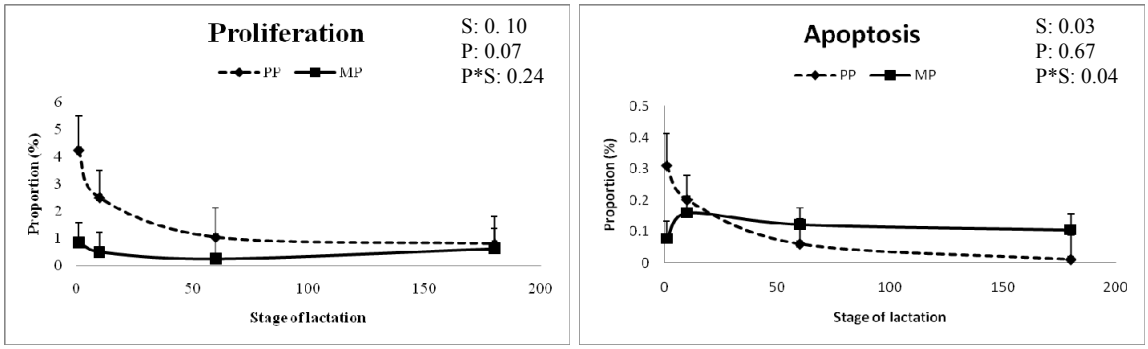
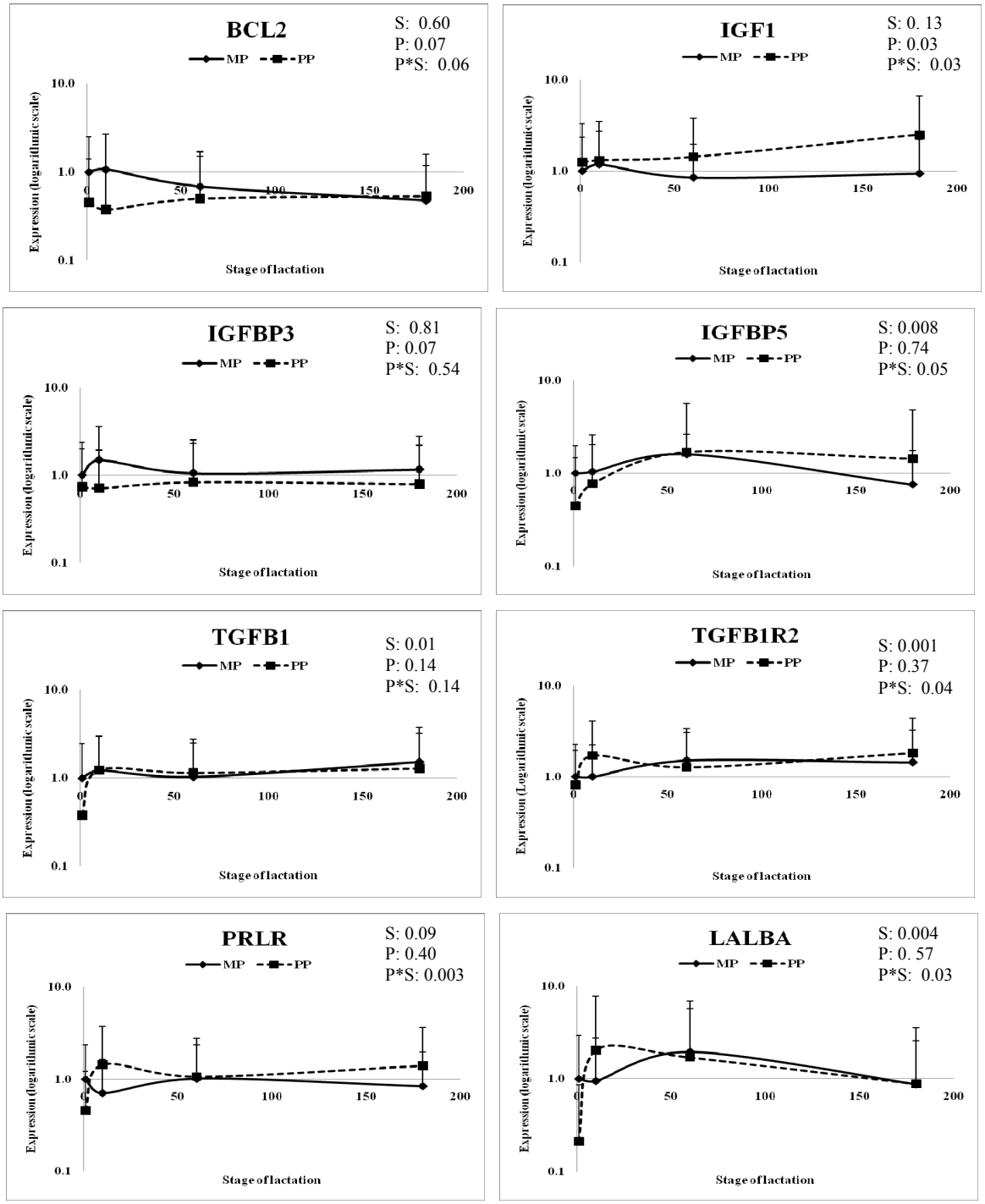
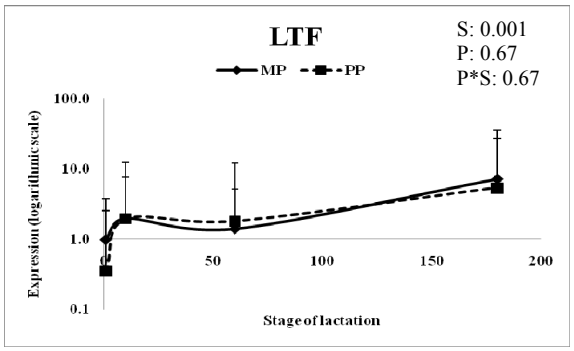
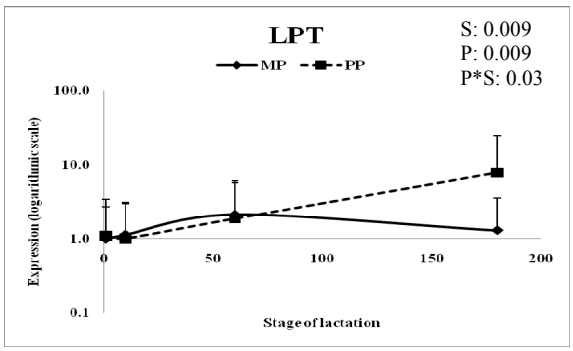

Proliferation
S: 0. 10
P: 0.07
P*S: 0.24
PP
MP
Proportion (%)
Stage of lactation
Apoptosis
S: 0.03
P: 0.67
P*S: 0.04
PP
MP
Proportion (%)
Stage of lactation

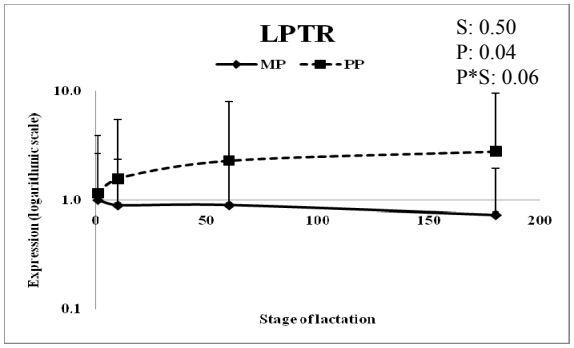
Figure 3.

A)

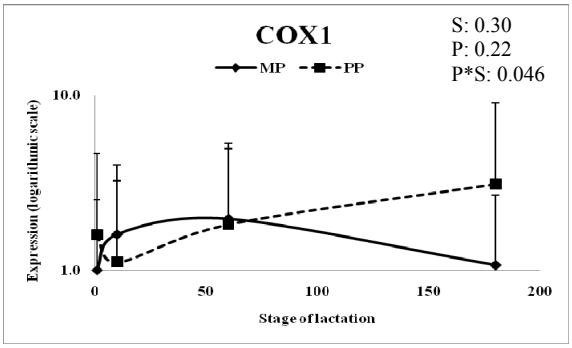

BCL2
S: 0.60
P: 0.07
P*S: 0.06
IGF1
S: 0. 13
P: 0.03
P*S: 0.03
IGFBP3
S: 0.81
P: 0.07
P*S: 0.54
IGFBP5
S: 0.008
P: 0.74
P*S: 0.05
TGFB1
S: 0.01
P: 0.14
P*S: 0.14
TGFB1R2
S: 0.001
P: 0.37
P*S: 0.04
PRLR
S: 0.09
P: 0.40
P*S: 0.003
LALBA
S: 0.004
P: 0. 57
P*S: 0.03
MP
PP
Expression (logarithmic scale)
Stage of lactation
10.0
1.0
0.1
0
50
100
150
200

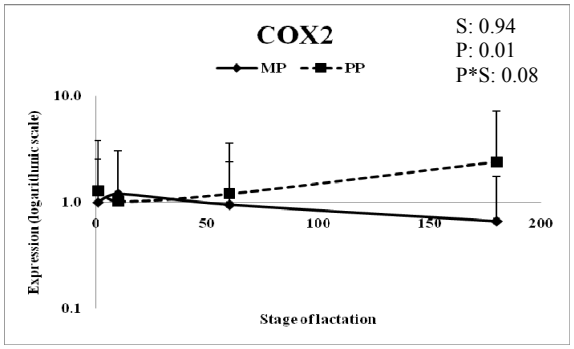

LTF
S: 0.001
P: 0.67
P*S: 0.67
MP
PP
Expression (logarithmic scale)
Stage of lactation

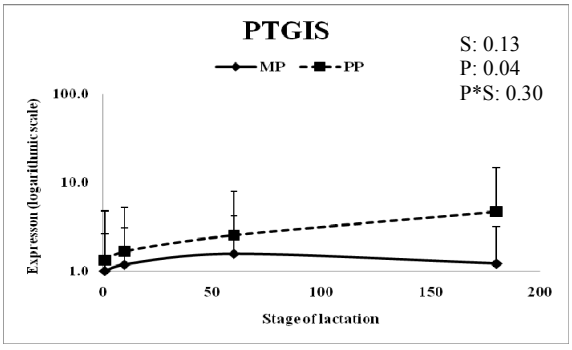

LPT
S: 0.009
P: 0.009
P*S: 0.03
MP
PP
Expression (logarithmic scale)
Stage of lactation

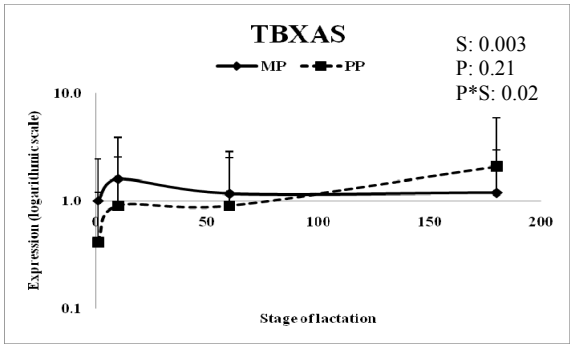

LPTR
S: 0.50
P: 0.04
P*S: 0.06
MP
PP
Expression (logarithmic scale)
Stage of lactation

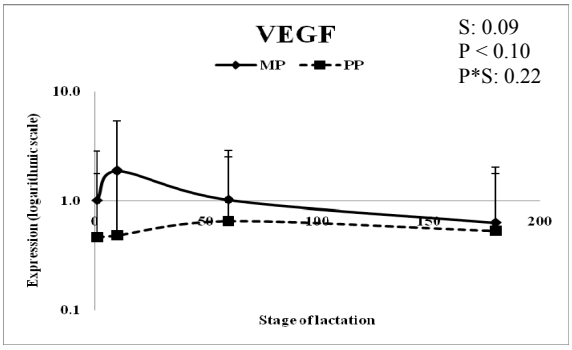
B)

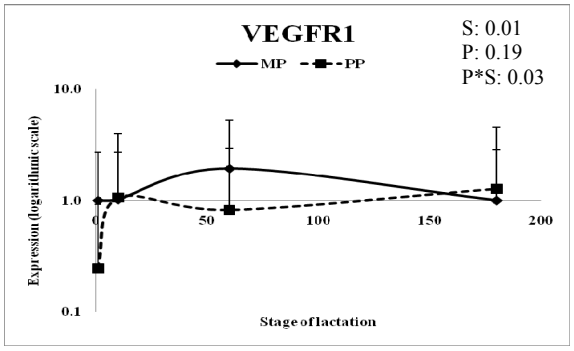

COX1
S: 0.30
P: 0.22
P*S: 0.046
MP
PP
Expression (logarithmic scale)
Stage of lactation

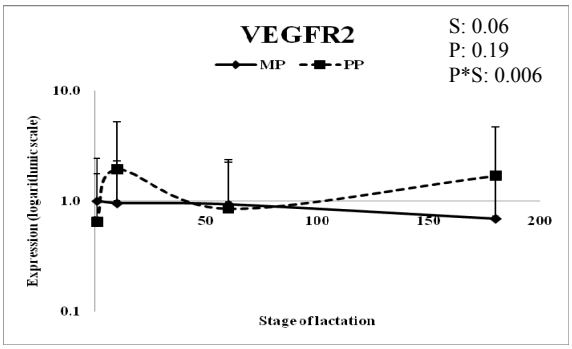

COX2
S: 0.94
P: 0.01
P*S: 0.08
MP
PP
Expression (logarithmic scale)
Stage of lactation

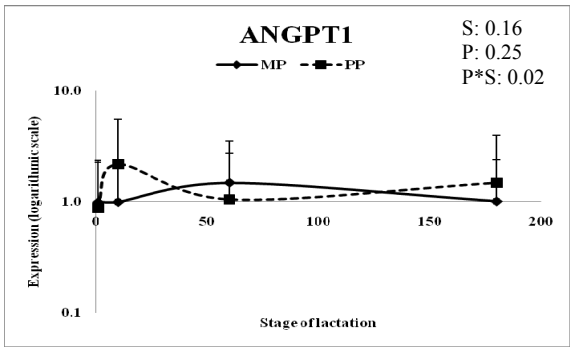

PTGIS
S: 0.13
P: 0.04
P*S: 0.30
MP
PP
Expression (logarithmic scale)
Stage of lactation

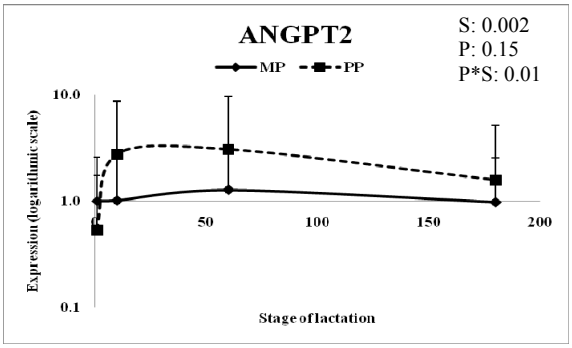

TBXAS
S: 0.003
P: 0.21
P*S: 0.02
MP
PP
Expression (logarithmic scale)
Stage of lactation

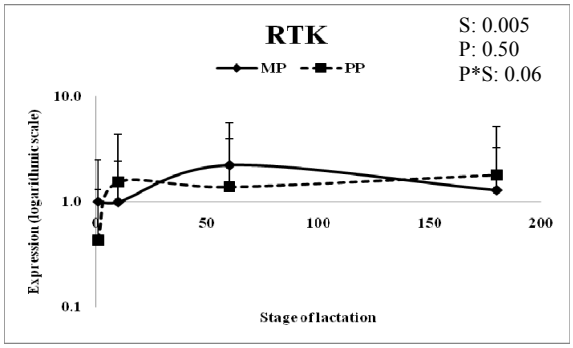

VEGF
S: 0.09
P < 0.10
P*S: 0.22
MP
PP
Expression (logarithmic scale)
Stage of lactation


VEGFR1
S: 0.01
P: 0.19
P*S: 0.03
MP
PP
Expression (logarithmic scale)
Stage of lactation


VEGFR2
S: 0.06
P: 0.19
P*S: 0.006
MP
PP
10.0
1.0
0.1
50
100
150
200
Expression (logarithmic scale)
Stage of lactation


ANGPT1
S: 0.16
P: 0.25
P*S: 0.02
MP
PP
10.0
1.0
0.1
0
50
100
150
200
Expression (logarithmic scale)
Stage of lactation


ANGPT2
S: 0.002
P: 0.15
P*S: 0.01
MP
PP
10.0
1.0
0.1
50
100
150
200
Expression (logarithmic scale)
Stage of lactation


RTK
S: 0.005
P: 0.50
P*S: 0.06
MP
PP
10.0
1.0
0.1
50
100
150
200
Expression (logarithmic scale)
Stage of lactation

PAPER 4:

Intravenous Supplementation of Acetate, Glucose or Essential Amino Acids to an Energy and Protein Deficient Diet in Early and Late Lactating Dairy Goats: Effects on Milk Production and Mammary Nutrient Extraction

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To be submitted to Journal of Dairy Science.

ABSTRACT

The objectives of the present experiment were to study how mammary supply of essential amino acids versus energy yielding substrates in the form of acetate or glucose would affect mammary nutrient uptake and milk (protein) synthesis in early (**EL**) and late lactating (**LL**) dairy goats. Goats were fed a basal diet deficient in energy (90% of requirements) and protein (80% of requirements), and were randomly allocated to 4 treatments in a balanced 4 x 4 Latin Square design. The treatments consisted of 4-d continuous intravenous infusions of isoosmotic isoenergetic solutions of essential amino acids (**EAA**), sodium acetate (**ACE**) and glucose (**GLU**) with saline (**SAL**) as control. There was a 3-d rest period between each treatment. Simultaneous arterio-venous blood samplings over each udder half (gland) were performed every 4 h during the last 24 h of infusion. Blood acid-base parameters and plasma concentrations of glucose, non-esterified fatty acids, beta-hydroxy-butyrate, triacylglycerol and urea were determined. Milk production over the last 48 h of infusion was recorded, and milk fat and protein contents determined. Milk yield and energy corrected milk, milk fat and protein yields in g/last 48 hours were significantly higher in EL than LL. Gross milk yield was significantly higher on GLU and EAA treatments in EL compared to the SAL control treatment, and yield of ECM was increased on ACE relative to SAL. In LL, nutrient supplementations did not affect gross milk yield, but ECM was increased on EAA compared to other treatments. Protein percentage was lowest on GLU compared to other treatments, and protein yields were increased on EAA and ACE but not on GLU compared to SAL in EL. In LL, milk protein percentage was higher on EAA than GLU and milk protein yield was higher on EAA compared to ACE and GLU. Fat percentages were decreased by GLU and EAA compared to ACE and SAL in EL, and highest fat yield in EL was observed on ACE. In LL, GLU also lowered milk fat percentage, whereas EAA increased it in contrast to the effect in EL. We conclude that an insufficient amino acid supply to the mammary gland of dairy goats can be compensated in EL but perhaps not LL by increased mammary supply and uptake of an energy yielding substrate, provided this substrate specifically contributes to ATP generation in mammary epithelial cells. The mammary gland may thus be relatively less sensitive towards variations in amino acid supply in EL compared to LL. This suggests there might be scope for development of differential protein recommendations for ruminants in early and late lactation, and this issue should be pursued in future studies.

Key words: mammary metabolism, mammary nutrient uptake, arterio-venous concentration difference, acid-base parameters

INTRODUCTION

Consumers' increased demand for protein has promoted the value of milk protein production over the last decades. Milk protein synthesis in dairy cows has been shown to be stimulated by increased amino acid supply either through dietary protein supplementation (Hanigan et al., 2001; Rulquin et al., 1993; Weekes et al., 2006) or by intravascular infusion of amino acids (Kim et al., 2000; Metcalf et al., 1996). However, increased mammary supply of a number of specific amino acids have not always had a positive impact on milk protein production (Seymour et al., 1990). Synthesis of protein in the mammary gland as well as in other body tissues relies not only on building blocks in form of amino acids, but also on availability of energy in the form of ATP. Raggio et al. (2006) therefore suggested that milk protein production could be improved by protein as well as energy supply to the mammary gland. Thus, intravascular infusion of energy yielding substrates like acetate (Chaiyabutr et al., 1998; Maas et al., 1995; Weekes et al., 2006) and glucose (Bobe et al., 2009; Schei et al., 2007) has been shown to affect milk protein synthesis positively.

Acetate and glucose are the major energy suppliers to the mammary gland. Acetate is utilized in oxidative phosphorylation of adenosine nucleosides, which results in the generation of ATP (Forsberg et al., 1984; Scott et al., 1976), whereas glucose is oxidized mainly through the pentose phosphate pathway to yield NADPH required for *de novo* fatty acid synthesis (lipogenesis) (Chaiyabutr et al., 1980; Chaiyabutr et al., 2008). If mammary energy supply is a main determinant for protein synthesis; it should theoretically be possible to substitute amino acids to some extent in the diet with an energy yielding susbstrate to provide extra ATP in the mammary gland and improve utilisation of the amino acids available.

With progressing lactation, the milk production efficiency decline and the milk composition in dairy animals change as a result of declined number and activity of the secretory cells in the mammary tissue (Safayi et al., 2009; Wilde and Knight, 1989). Along with these changes, mammary nutritional requirements and uptake are also changed (Aganga et al., 2002; Nielsen et al., 2001). There are indications suggesting that this is associated with an altered sensitivity of the mammary gland towards variations in e.g. amino acid supply, since correlations between arterial concentrations and arterio-venous differences (efficiency of mammary uptake) for individual amino acids differed in early compared to late lactation in dairy goats fed different levels of lysine and methionine (Madsen et al., 2005). Therefore, it is also likely that there might be a prospect for differentiating protein recommendations for ruminants across the lactation period, and still maintain protein production by optimisation of the provision of energy yielding substrates relative to amino acids (Madsen et al., 2005).

We hypothesised that mammary supply of essential amino acids versus energy yielding substrates in the form of acetate or glucose would affect mammary nutrient uptake and milk (protein) synthesis in both early and late lactating dairy goats that were fed a basal diet deficient in energy (90% of requirements) and protein (80% of requirements). The more specific aims of the present project were to determine whether : 1) provision of energy (ATP) yielding substrates can compensate for an insufficient AA supply to the mammary gland and hence improve overall utilization of AA for milk protein synthesis, 2) acetate is more efficient than glucose in stimulating milk synthesis and particularly its milk protein content due to their contribution to mainly ATP and NADPH, respectively; and 3) milk synthesis is more sensitive towards changes in nutrient provision in early lactation (**EL**) compared with late lactation (**LL**). This issue is of major importance for the economy of dairy production by increasing our knowledge on how to maximize not only the milk protein yield but also the overall nitrogen utilization in dairy farming.

The objectives were addressed in a study with goats conducted in both early and late lactation. The goats were fed a basal ration designed to meet approximately 90% and 80% of daily requirements for net energy and amino acids absorbable from the small intestine, respectively, and given intravenous infusions of isoosmotic solutions of essential amino acids (**EAA**), acetate (**ACE**), glucose (**GLU**) and saline (**SAL**) as control in a 4 x 4 Latin square design.

MATERIALS AND METHODS

Experimental design

Four Danish Landrace dairy goats (parity ≥ 2) were used in this experiment. Goats were previously surgically prepared with exteriorized carotid arteries and milk veins, as described by Nielsen et al. (1995). The goats were randomly assigned to one of four treatments in a balanced 4 x 4 Latin square design, which was performed in both late lactation (**LL**) and the following early lactation (**EL**). Due to health reasons, one of the goats had to be replaced in the early lactation trial with her twin sister, which had a similar size, body weight and milk yield. At the start of each part of the experiment, early and late lactation, the goats were 21±1 and 157±9 days postpartum, and live weights were 51±8 and 52±3 kg, respectively.

Treatments consisted of continuous intravenous infusions of 4 isoosmotic solutions at pH 7.4 (960 g/d for 4 days) containing either saline (**SAL**) as control, a mixture of essential amino acids (**EAA**) composed to match the relative proportion of EAA in milk protein determined in a previous experiment with this goat breed (Madsen et al., 2005), sodium acetate (**ACE**), or glucose (**GLU**). The chemical composition of the feed used in both early and late lactation as

well as an overview of the daily provision of energy and protein to the goats with the daily diet and infusion solutions are shown in tables 1 and 2. EAA, ACE and GLU infusions were composed to be isoenergetic based on the potential yield of ATP from oxidation of these nutrients in the body (33.6, 10 and 36 mol ATP/mol EAA mix, ACE and GLU, respectively). Each treatment period lasted one week, where the intravenous infusions were initiated at 12:00 on day 1 (Monday) and concluded at 12:00 on day 5 (Friday), followed by a 3 day rest period in between. Samplings were performed during the last 24 hours of each infusion period. The goats were milked and fed the restricted basal diet in two daily meals (at 07:00 and 17:00 h) during the first 3 days of each infusion period, and every 4 hours for the last 24 hours during the sampling period. The diet (see Table 2) was consisted of artificially dried grass hay, barley, protected fat, urea, and a vitamin/mineral mix. It was designed to meet approximately 90% and 80% of daily requirements for energy and amino acids absorbable from the small intestine, respectively, based on the pre-trial body weight and milk yields in the early and late lactation period and according to Danish feeding standards (Strudsholm et al., 1999). The Danish standards are consistent with the nutrient requirements of dairy goats, as reported by Sauvant and Morand-Fehr (1989).

The experiment was carried out at the experimental facilities at the Faculty of Life Sciences, University of Copenhagen, Denmark, and approved by the Danish Animal Experimentation Inspectorate, and complied with the Danish Ministry of Justice laws concerning animal experimentation and care for experimental animals.

Sampling

During each treatment period, the goats were placed in metabolic cages and the daily feed intake was determined. Feed refusals from the last 24 hours of each infusion period were collected. Milk yields were recorded at each milking. As described by Madsen et al. (2005), catheters were fitted in one carotid artery using local analgesic (20 mg/mL lidocain, AstraZeneca, Albertslund, Denmark) and in both milk veins. Blood samples from the carotid artery, and the milk veins (whilst manually clamping the vena pudenda externa) were withdrawn simultaneously seven times (every 4 hours) during the last 24 h period of each treatment starting at 12:00 on day 4 and ending at 12:00 on day 5. Blood samples were taken using 1 ml LiHep coated syringes (PICO50, Radiometer, Denmark) for immediate determination of acid-base parameters. Other blood samples were collected in 10 ml vacutainers coated with EDTA, and stored on ice till centrifugation (3000 gav at 4 °C for 12

min). Plasma was transferred to labeled cryo-tubes. Samples of the offered feed, feed refusals and milk (from each milking over the last 24 h) were collected and stored at -20 ºC.

Analyses and Calculations

Blood samples collected with 1 ml LiHep coated syringes, were immediately analyzed for acid-base parameters: pH, $HCO3^-$, total O_2 (**TO2**) and total CO_2 (**TCO2**) (ABL700SERIES, USA and GEM OPL, USA). Stored plasma samples were analyzed for glucose (GLU), non-esterified fatty acids (**NEFA**), beta-hydroxy-butyrate (**BHB**), triglycerol (**TG**) and urea. As described by Husted et al. (2008), all analyses were performed using an autoanalyzer, ADVIA 1650® Chemistry System (Bayer Corporation, Tarrytwon, NY 10592, USA), at the Faculty of Agricultural Sciences, Aarhus University, Denmark. Plasma content of total long-chain fatty acids (**LCFA**) was calculated as concentration of NEFA + 3 x concentration of TG. Plasma concentration of IGF-1 was determined in the first arterial sample on every sampling day, as described by Tygesen et al. (2008). A commercial spectrophotometric kit was used for determination of plasma concentration of acetate (ACE) (AnzyPlus Rasio Diagnostic A/S, Italy). Milk protein and fat content was analyzed and calculated as described by Nielsen and Jakobsen (1993). Mammary extraction (**E**) rate for metabolites was calculated as mammary arterial-milk vein concentration difference (**AVD**) divided by arterial concentration. Respiratory quotient (**RQ**) values for each mammary gland was calculated as mammary CO_2 release divided by mammary O_2 uptake = (TCO2 (mM) x mammary blood flow) / (TO2 (mM) x mammary blood flow) = TCO2 AVD / TO2 AVD. Energy corrected milk yield (**ECM**) was calculated as milk yield (g/last 48 hours) x ((0.383 x fat% + 0.242 x protein% + 0.7832) / 3.14), where 1 g of ECM equals 3.14 kJ (Sjaunja et al., 1991).

Statistical Analysis

Statistical evaluation of all data was performed using the Mixed procedure in SAS (v.9.1; SAS Institute, USA). Variables in the statistical models included treatment, experiment week and the time at which the samples were taken, the experimental stage and its interaction with treatment as fixed effects, and the factor gland within goat was considered as random effect, as shown in the following model:

$$Y = \mu + \alpha_i + \beta_j + \gamma_k + \lambda_l + \eta_{il} + \rho_m + \tau_n + \varepsilon_{ijklmn}, \text{ where}$$

Y = the dependent variables, μ = the overall means, α_I = treatment (I = ACE, EAA, GLU or SAL), β_j = experimental week (j = 1, 2, 3 or 4), γ_k = sampling time (k= 1, 2, 3, 4, 5, 6 or 7), λ_l

= lactation stage (l = EL or LL), η_{il} = interaction between treatment and lactation stage, ρ_m = random effect of goat (m =1, 2, 3 or 4), τ_n = random effect of gland (n = left or right) and ε_{ijklm} = random variation which was assumed to be normally distributed with a variance σ^2 and a mean of zero. In addition, statistical analyses were performed separately for each individual stage of lactation for milk yield data to compare the treatment effects within each stage more precisely. Before performing the final statistical analyses, data were checked for outliers based on residual plots. Presented results are expressed as least squares means (LS-Means) with standard error of mean (**SEM**). One goat went off her feed in the last experimental week in LL on the ACE treatment and showed extreme values for most of the blood parameters. Her data for that week was taken out of the final statistical analyses. The PDIFF option in SAS was used to generate comparisons between treatment means. The level of significance was set at $P < 0.05$.

RESULTS

Milk Yield and Composition

In the following the changes in EL and LL in milk yield, energy corrected milk (**ECM**), percentages and yields of both milk fat and protein will be presented for the three different nutrient infusions compared to SAL as control infusion. Statistical analyses were performed separately for each stage of lactation. All the results are shown in Table 3.

Milk yield. Gross milk yield tended to be affected by treatment in EL (P = 0.06). GLU had a stimulating effect on milk yield compared to the control treatment (P = 0.01), and this also tended to be the case for EAA (P = 0.06) but not for ACE (P = 0.11). There was no effect of treatment on milk yield in LL. ECM was not affected by treatment in EL (P = 0.19), although ECM yields on ACE tended to be higher compared to SAL (P = 0.06). In LL, however, ECM was affected by treatment (P=0.03), and the highest ECM yield was observed in response to EAA infusion compared to GLU (P = 0.01), ACE (P = 0.09) and SAL (P = 0.06). Both milk yield and ECM were significantly higher in EL than LL.

Milk protein. Milk protein percentage was generally lower but total protein yield higher in EL compared to LL, for all treatments except ACE, where milk protein percentage was the same in EL and LL (P = 0.18). In EL, milk protein percentage was significantly lower on GLU compared to other treatments including the control group (P = 0.03). Milk protein yield was increased by provision of EAA (P = 0.03) compared to the SAL control, and provision of ACE (P = 0.08) also tended to raise milk protein yield above that on the control treatment. In

LL, milk protein percentage was significantly higher on EAA compared to GLU (P = 0.03), and milk protein yield was higher on EAA compare to GLU (P < 0.01) and tended to be higher compared to ACE (P = 0.06).

Milk fat. In EL, milk fat percentages were similar on ACE and SAL treatments and significantly higher compared to EAA and GLU, and fat yield on ACE tended to be higher than on EAA (P = 0.07). In LL the picture was slightly different. Fat percentage was still lowest on GLU but not significantly different from SAL (P = 0.17), whereas highest fat percentages were found on ACE and also EAA compare to GLU and SAL treatments. Compared with SAL, fat yield was increased by EAA and decreased by GLU in LL (P = 0.06 and P = 0.01, respectively). Milk fat percentage was at the same level in EL as in LL, but fat yield was higher in EL for all treatments.

Arterial Concentrations (A), Arterial-Milk Vein Concentration Differences (AVD), and Mammary Extraction Rates (E) of Plasma Metabolites

In the following the changes in A, AVD and E of plasma metabolites across the mammary gland will be presented for the three different nutrient infusions compared to SAL as control infusion in both EL and LL. All the results are shown in Table 4.

Glucose: A was highest on GLU compared to other treatments in both EL and LL. In EL, glucose E was lower on GLU compared to EAA treatment, and glucose AVD was higher on EAA compared to ACE. In LL, the lowest GLU E and AVD was observed on GLU compared to all other treatments. AVD and E rates for glucose were higher in EL compared to LL for all treatments except SAL.

Acetate: A and AVD was either numerically or significantly higher on ACE treatment compared to other treatments in both EL and LL. AVD changed in proportion to A, so that acetate E remained constant across treatments in both EL and LL. However, acetate E was affected by stage of lactation, being higher in EL compared to LL across all treatments (EAA: P <0.01, GLU:P < 0.001, SAL: P= 0.07) except ACE (P = 0.41).

BHB: A, AVD in EL and LL as well as its E rate in LL were lowest on GLU compared to all other treatments and lower for EAA and ACE compared to SAL. Although the A concentrations for all treatments were lower in EL than LL, the E rates were higher.

NEFA: A and AVD in EL were lower on GLU compared to other treatments, and lower for EAA and ACE compare to SAL. In LL, both A and AVD was highest on EAA compared to all other treatments. E rates were not affected by treatment in neither EL nor LL. A, AVD and E rates for NEFA were, however, significantly higher in EL than LL except for A and AVD on EAA treatment.

TG and LCFA: A and AVD were unaffected by any of the treatments in EL, but E ratios were significantly lower on SAL control compared to all other treatments. In LL, A was highest on SAL and AVD and E were lowest on GLU compared to the other treatments. In EL compared to LL, A for TG and LCFA were generally higher (all treatments), AVD lower (on ACE and SAL treatments) and E also lower (all treatments except for SAL).

Urea: A was highest on ACE compared to other treatments in EL, but highest on EAA compared to other treatments in LL.AVD as well as E rates were not significantly different from zero and are not shown.

IGF1 and insulin: A concentrations were neither affected by treatment nor by stage of lactation.

Arterial Concentrations (A), Arterial-Milk Vein Concentration Differences (AVD), and Mammary Extraction Rates (E) of Acid-Base Parameters

In the following the changes in A, AVD and E for blood acid-base parameters will be presented for the three different nutrient infusions compared to the SAL control infusion in both EL and LL. All the results are shown in Table 5.

TO_2: In EL, A was unaffected by treatment, but AVD and E were highest on EAA compared to other treatments, and E was higher on ACE compare to GLU. In LL, A, AVD and E were lower on EAA, ACE and GLU treatments compared to SAL. A AVD and E for TO_2 were not affected by stage of lactation, except on SAL where the lowest values were observed in EL. EAA and E for ACE were higher.

TCO_2 and HCO_3: These two parameters changes in parallel. A was highest and E least negative on ACE, and AVD least negative on GLU compared to the other treatments in both EL and LL. A and E rate on GLU treatment was higher than on EAA and SAL in EL. In EL compare to LL, A was lower on EAA but higher on ACE, while its AVD and E rates were higher on ACE and SAL.

RQ: across the mammary gland was unaffected by treatment, but tended ($P = 0.09$) to be lower in EL compare to LL on all treatments.

pH: The highest arteral pH was found on ACE compared to other treatments in both EL and LL, whereas the lowest pH was found on EAA in EL and on EL and SAL in LL. Lactation stage effect was only seen on SAL, where pH was higher in EL compared to LL.

There was a week effect for many of the blood parameters, which probably reflects the changes in metabolic state goats would normally be experiencing during early lactation. There was also an effect of sampling time during the day of sampling for some acid-base parameters as well as metabolites. However, changes were not in any way systematically related to times

of feeding or milking, and were therefore considers to be random without biological significance in this context.

DISCUSSION

The general condition and behavior of the goats used in this experiment were normal on different treatments in both EL and LL, except the week they were getting ACE infusion. ACE was infused at constant pH (7.4), but during oxidation in the body, the infused acetate anions would associate with protons (H^+) resulting in loss of acid from the body resulting in a more alkalotic state. Thus, on the ACE treatment arterial blood pH, total CO_2 and HCO_3^- increased, reflecting a shift to the left in the carbonic acid buffering system in blood: $H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow CO_2 + H_2O$. The goats did not seem to be affected by this, judged from their overall behavior, except for one goat (who was in her final experimental week), and she was subsequently taken out of the experiment as previously described.

Substitution of essential amino acid supply to the mammary gland by energy yielding substrates and the effect on milk synthesis and composition

Continuous intraruminal infusion of acetate in different stages of lactation in dairy cows (Rook and Balch, 1961) and dairy goats (Lough et al., 1983) have been reported to increase milk fat yields, without any effect on neither milk volume nor protein yield. In the present study, milk fat percentage and yield were highest on ACE treatment in EL. This was expected since ACE contributes to milk fat synthesis as a substrate in *de novo* fatty acid synthesis (Bickerstaffe et al., 1974). Increased mammary uptake of acetate in response to increased supply can drive up milk fat, consisting of more short and medium chained fatty acids (Bauman and Griinari, 2003). Acetate is commonly believed to be taken up by the mammary gland in proportion to the concentration gradient across the mammary epithelial cell membrane, ie. in proportion to concentrations in arterial blood. AVD across the mammary gland for acetate was therefore increased to the highest levels during ACE treatment, when also A levels of acetate for obvious reasons increased. The additional mammary uptake of acetate on the ACE treatment did apparently not induce changes in the use of other fat precursors like BHB and LCFA, which agrees with previous findings (Purdie et al., 2008). Mammary E for acetate remained constant across the different treatments. However, E was higher in EL compared to LL, suggesting either that a higher mammary metabolic activity in EL can drive a higher uptake of acetate through lowering of intracellular acetate concentrations (pull effect) or that a higher proportion of mammary blood flow is directed

through arterio-venous shunts as lactation progresses, and not contributing to capillary (nutritive) perfusion (Fleet and Mepham, 1983).

A milk protein reduction with intravascular GLU infusion has previously been reported in dairy cows by Cant et al. (2002), and Hurtaud et al. (1998; 2000) reported in dairy cows that intra-duodenal infusion of GLU did not lead to an increase in milk protein yield. The decrease in protein and fat percentages in EL on GLU treatment could most probably be ascribed to a dilution effect explained by increased lactose synthesis in response to the provision of glucose (Cant et al., 2002; Rook, 1979). There was a significant increase in gross milk yield and a numerical increase in ECM in EL but not in LL in response to GLU infusion. This would be in agreement with the mammary gland being capable of increasing glucose uptake to some extent in EL in response to increased supply, but not as efficiently in LL where the synthetic capacity of the mammary gland is presumed to be the most limiting factor for glucose uptake (Nielsen et al., 2001). Infusion of GLU in LL did therefore presumably not lead to increased mammary uptake, but rather increased uptake of nutrients in non-mammary tissues, and this could have been associated with a general diversion of nutrients towards non-mammary metabolism, as indicated by the reduction in milk fat content and yield during GLU treatment in LL. However, insulin and IGF-1 plasma levels were surprisingly not significantly affected by GLU treatments in neither EL nor LL despite elevation of plasma glucose levels. The highest NEFA levels, however, were observed on the SAL treatment in EL, indicating that experimental animals in EL would have been in the most negative energy balance on this treatment, when fed the protein and energy deficient basal diet without any additional supplementation. This may in turn explain why fat percentage in milk in EL on the SAL treatment was as high as on the ACE treatment.

Compared to the SAL control treatment, milk protein yield was increased by provision of EAA and an energy yielding substrate in the form of ACE in EL. But in LL, insufficient mammary EAA supply could apparently not be compensated as efficiently by provision of more energy-yielding substrates. Acetate and glucose are both substrates contributing to oxidative metabolism in the mammary epithelial cell, but acetate is quantitatively the most important of the two in both dairy goats and cows (Bickerstaffe et al., 1974). Glucose, has a reported entry rate in the order of 52-70 % of that of acetate (Bickerstaffe et al., 1974). Oxidation of acetate is coupled to oxidative phosphorylation of adenosine nucleosides, which results in the generation of ATP (Forsberg et al., 1984; Scott et al., 1976), whereas glucose in the mammary gland is oxidized mainly through the pentose phosphate pathway to generate NADPH required for *de novo* fatty acid synthesis (lipogenesis) (Chaiyabutr et al., 1980;

Chaiyabutr et al., 2008). Milk protein synthesis is a highly energy consuming process, and 5 ATP are consumed for every peptide bond formed (Hanigan and Baldwin, 1994; Lobley, 1990). Our results showed that it is possible in EL to sustain milk protein synthesis whilst reducing amino acid supply to the mammary gland provided: 1) that the mammary supply and uptake of energy yielding substrates are increased, and 2) that this additional energy supply is in the form of a nutrient (acetate) that contributes predominantly to ATP generation in the mammary epithelial cells. But when the energy supply is given in the form of glucose, protein synthesis and milk protein content can not be sustained, neither in EL nor in LL. This illustrates the importance of ATP for sustenance of milk protein synthesis.

Impact of stage of lactation on sensitivity of milk synthesis towards changes in nutrient provision

In EL, the synthetic capacity of the mammary gland is at its maximum and feed intake capacity normally not sufficient to cover the nutritional requirements for milk production. It is therefore generally anticipated that nutrient supply to the mammary gland is the main limiting factor for milk production in EL, whereas synthetic capacity becomes limiting later in lactation, when mammary epithelial cells die and feed intake increases to the extent where nutrient supply no longer limits nutrient uptake in the mammary gland (Madsen et al., 2005; Wilde and Knight, 1989). With progressing lactation, it therefore appears logical to assume that milk synthesis within the mammary gland should become less sensitive to changes in supply of nutrients. In LL, additional supply of acetate did indeed fail to drive up milk fat synthesis, although AVD for acetate increased on ACE compared to all other treatments. This indicates that mammary gland synthetic capacity was the main limiting factor for *de novo* fatty acid synthesis at this stage of lactation and not substrate supply. Milk TG synthesis may however not be limited by changes in synthetic capacity to the same extent as *de novo* fatty acid synthesis in LL, since a very high positive correlation ($R2 < 0.01$) was found between milk fat yield and AVD for NEFA in LL.

In LL, the population of transporters in the MEC for both glucose (Shennan and Peaker, 2000) and amino acids (Sharma and Kansal, 2000) decrease compared to EL. The same has been reported for activity of the capillary enzyme lipoprotein lipase involved in mammary uptake of plasma TG (Neville and Picciano, 1997). Hence more nutrients provided by the blood will escape mammary uptake. Consequently AVD and E were significantly lower in LL compared to EL, reflecting the decreased synthetic capacity and metabolic activity in the organ, with a decreasing capability to extract nutrients from whatever volume of blood that flows through it. Even for nutrients where concentration gradients are believed to be the

driving force for mammary uptake (acetate, BOHB and NEFA), efficiency of mammary uptake decreased as lactation progressed, showing that extraction of such nutrients is determined not only by arterial concentrations, but probably also by the intracellular concentration and hence metabolic activity in the mammary epithelial cells.

Quantitative nutrient uptake in the mammary gland is determined by AVD as well as the rate of MBF. According to Cant et al. (2003), amino acids are not directly involved in regulation of vascular tone and hence MBF. The majority of published studies have shown a positive or no effect of increasing amino acid supply to the mammary gland on MBF. Thus MBF was neither in early nor in late lactating dairy goats affected by dietary supplementation with protected Lys and Met (Madsen et al., 2005). In dairy cows, close-arterial infusion into the mammary gland of a mix of 18 AA excluding Leu was reported to increase MBF (Bequette et al., 1996), and it was suggested by Cant et al (2003) that MBF and net extraction of deficient amino acids are elevated during a single amino acid deficiency, by which mammary blood flow increases. But in one study (Guinard and Rulquin, 1995), duodenal infusions of DL-Met in increasing doses has been reported to have a negative impact on MBF. Rulquin et al. (2004) reported that intra-duodenal infusion of glucose in dairy cows increased MBF. In other studies in dairy cows a negative impact of glucose infusion on illiac plasma flow has been found (Cant et al., 2002; Purdie et al., 2008), but to what extent the decreased iliac flow could be ascribed to decreased MBF or decreased blood supply to the hind leg is unknown. We did not measure mammary blood flow in this experiment, but it seems from previous studies that mammary blood flow would most likely have been unaffected or if anything increased on EAA and GLU treatments. Yet, there was no indication of a positive milk yield response on ACE treatment in LL. Only supplementation of additional EAA to the basal energy and protein deficient diet could drive up synthesis of ECM, milk protein as well as fat in LL. This milk yield response was associated with increased uptake of NEFA (high AVD across the mammary gland) due to increased arterial levels of NEFA, and urea levels were also increased in LL. We have no explanation why the EAA treatment apparently induced a more catabolic state in the animals compared to the SAL control.

In a previous goat experiment we showed that AVD for lysine and methionine across the mammary gland increased linearly with A in both EL and LL in response to increased dietary supply. But it required higher arterial concentrations to drive a given uptake (AVD) in LL (Madsen et al., 2005). It has been shown by (Sharma and Kansal, 2000) in the rabbit that the relative abundance of different amino acid transporters in the mammary epithelial cell membrane changes during lactation. This observation along with our results, suggest that the

mammary gland in EL may in fact be more robust towards changes in amino acid supply, capable of maintaining amino acid uptake even at rather low arterial concentrations. And this may explain why an insufficient amino acid supply in EL could be compensated by a larger energy (ATP) supply, whereas this was not possible in LL.

CONCLUSION

Milk protein synthesis requires presence in the mammary epithelial cell of building blocks in the form of amino acids as well as energy in the form of ATP. An insufficient supply of amino acids to the mammary gland of dairy goats can be compensated in EL but perhaps not LL by increased energy supply to the mammary gland, provided that this increased energy supply is in the form of nutrients like acetate, which are metabolized predominantly in pathways resulting in generation of ATP. The mammary gland may thus be relatively less sensitive towards variations in amino acid supply in EL compared to LL, but the underlying reason for this altered sensitivity during lactation remains to be established. The present study thus indicate that there could be scope for development of differential protein recommendations for ruminants in early and late lactation, and this issue should be pursued in future studies.

ACKNOWLEDGMENTS

This project was financed by the Danish Research Council for Technology and Production Sciences. Sina Safayi was in receipt of a PhD scholarship co-financed by the Danish Research Council and Mrs. M. Namian. The authors would like to acknowledge V.G. Christensen, D.S. Jensen, R. Jensen, B. Dahl, E. de N. Harrison, Merete Stubgaard and Inge Mejdahl for their valuable technical assistance. We also wish to express our gratitude to the experimental farm staff at the Faculty of Life Sciences, University of Copenhagen, Denmark.

REFERENCES

Aganga, A. A., J. O. Amarteifio, and N. Nkile. 2002. Effect of Stage of Lactation on Nutrient Composition of Tswana Sheep and Goat's Milk. Journal of Food Composition and Analysis 15(5):533-543.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annual Review of Nutrition 23:203-227.

Bequette, B. J., J. A. Metcalf, D. WrayCahen, F. R. C. Backwell, J. D. Sutton, M. A. Lomax, J. C. Macrae, and G. E. Lobley. 1996. Leucine and protein metabolism in the lactating

dairy cow mammary gland: Responses to supplemental dietary crude protein intake. Journal of Dairy Research 63(2):209-222.

Bickerstaffe, R., E. F. Annison, and J. L. Linzell. 1974. Metabolism of Glucose, Acetate, Lipids and Amino-Acids in Lactating Dairy-Cows. Journal of Agricultural Science 82(FEB):71-85.

Bobe, G., A. R. Hippen, P. She, G. L. Lindberg, J. W. Young, and D. C. Beitz. 2009. Effects of glucagon infusions on protein and amino acid composition of milk from dairy cows. J. Dairy Sci. 92(1):130-138.

Cant, J. P., D. R. Trout, F. Qiao, and N. G. Purdie. 2002. Milk synthetic response of the bovine mammary gland to an increase in the local concentration of arterial glucose. J. Dairy Sci. 85(3):494-503.

Cant, J. R., R. Berthiaume, H. Lapierre, P. H. Luimes, B. W. McBride, and D. Pacheco. 2003. Responses of the bovine mammary glands to absorptive supply of single amino acids. Canadian Journal of Animal Science 83(3):341-355.

Chaiyabutr, N., A. Faulkner, and M. Peaker. 1980. Utilization of Glucose for the Synthesis of Milk Components in the Fed and Starved Lactating Goat Invivo. Biochemical Journal 186(1):301-308.

Chaiyabutr, N., S. Komolvanich, S. Sawangkoon, S. Preuksagorn, and S. Chanpongsang. 1998. Glucose metabolism in vivo in crossbred Holstein cattle feeding on different types of roughage during late pregnancy and early lactation. Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology 119(4):905-913.

Chaiyabutr, N., S. Komolvanich, S. Thammacharoen, and S. Chanpongsang. 2008. Effects of long-term exogenous bovine somatotropin on glucose metabolism and the utilization of glucose by the mammary gland in different stages of lactation of crossbred Holstein cattle. Animal Science Journal 79(5):561-574.

Fleet, I. R., and T. B. Mepham. 1983. Physiology Methods Used in the Study of Mammary Substrate Utilization in Ruminants. Pages 469-491 in Biochemistry of Lactation. T. B. Mepham, ed. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Forsberg, N. E., R. L. Baldwin, and N. E. Smith. 1984. Roles of Acetate and Its Interactions with Glucose and Lactate in Cow Mammary Tissue. J. Dairy Sci. 67(10):2247-2254.

Guinard, J., and H. Rulquin. 1995. Effects of Graded Amounts of Duodenal Infusions of Methionine on the Mammary Uptake of Major Milk Precursors in Dairy-Cows. J. Dairy Sci. 78(10):2196-2207.

Hanigan, M. D., and R. L. Baldwin. 1994. A Mechanistic Model of Mammary-Gland Metabolism in the Lactating Cow. Agricultural Systems 45(4):369-419.

Hanigan, M. D., B. J. Bequette, L. A. Crompton, and J. France. 2001. Modeling mammary amino acid metabolism. Livestock Production Science 70(1-2):63-78.

Hurtaud, C., S. Lemosquet, and H. Rulquin. 2000. Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. J. Dairy Sci. 83(12):2952-2962.

Hurtaud, C., H. Rulquin, and R. Verite. 1998. Effects of level and type of energy source (volatile fatty acids or glucose) on milk yield, composition and coagulating properties in dairy cows. Reproduction Nutrition Development 38(3):315-330.

Husted, S. M., M. O. Nielsen, D. Blache, and K. L. Ingvartsen. 2008. Glucose homeostasis and metabolic adaptation in the pregnant and lactating sheep are affected by the level of nutrition previously provided during her late fetal life. Domestic Animal Endocrinology 34(4):419-431.

Kim, C. H., J. J. Choung, and D. G. Chamberlain. 2000. The effects of intravenous administration of amino acids and glucose on the milk production of dairy cows consuming diets based on grass silage. Grass and Forage Science 55(2):173-180.

Lobley, G. E. 1990. Energy-Metabolism Reactions in Ruminant Muscle - Responses to Age, Nutrition and Hormonal Status. Reproduction Nutrition Development 30(1):13-34.

Lough, D. S., E. C. Prigge, W. H. Hoover, and G. A. Varga. 1983. Utilization of Ruminally Infused Acetate Or Propionate and Abomasally Infused Casein by Lactating Goats. J. Dairy Sci. 66(4):756-762.

Maas, J. A., D. R. Trout, J. P. Cant, B. W. McBride, and D. P. Poppi. 1995. Method for Close Arterial Infusion of the Lactating Mammary-Gland. Canadian Journal of Animal Science 75(3):345-349.

Madsen, T. G., L. Nielsen, and M. O. Nielsen. 2005. Mammary nutrient uptake in response to dietary supplementation of rumen protected lysine and methionine in late and early lactating dairy goats. Small Ruminant Research 56(1-3):151-164.

Metcalf, J. A., L. A. Crompton, D. Wray-Cahen, M. A. Lomax, J. D. Sutton, D. E. Beever, J. C. Macrae, B. J. Bequette, F. R. C. Backwell, and G. E. Lobley. 1996. Responses in Milk Constituents to Intravascular Administration of Two Mixtures of Amino Acids to Dairy Cows. J. Dairy Sci. 79(8):1425-1429.

Neville, M. C., and M. F. Picciano. 1997. Regulation of milk lipid secretion and composition. Annual Review of Nutrition 17:159-183.

Nielsen, M. O., and K. Jakobsen. 1993. Changes in Mammary Glucose and Protein-Uptake in Relation to Milk Synthesis During Lactation in High-Yielding and Low-Yielding Goats. Comparative Biochemistry and Physiology A-Physiology 106(2):359-365.

Nielsen, M. O., T. G. Madsen, and A. M. Hedeboe. 2001. Regulation of mammary glucose uptake in goats: role of mammary gland supply, insulin, IGF-1 and synthetic capacity. Journal of Dairy Research 68(3):337-349.

Nielsen, M. O., C. Schleisner, K. Jakobsen, and P. H. Andersen. 1995. The effect of mammary O-2 uptake, CO2 and H+ production on mammary blood flow during pregnancy, lactation and somatotropin treatment in goats. Comparative Biochemistry and Physiology A-Physiology 112(3-4):591-599.

Purdie, N. G., D. R. Trout, D. P. Poppi, and J. P. Cant. 2008. Milk synthetic response of the bovine mammary gland to an increase in the local concentration of amino acids and acetate. J. Dairy Sci. 91(1):218-228.

Raggio, G., S. Lemosquet, G. E. Lobley, H. Rulquin, and H. Lapierre. 2006. Effect of casein and propionate supply on mammary protein metabolism in lactating dairy cows. J. Dairy Sci. 89(11):4340-4351.

Rook, J. A., and C. C. Balch. 1961. Effects of Intraruminal Infusions of Acetic, Propionic and Butyric Acids on Yield and Composition of Milk of Cow. British Journal of Nutrition 15(3):361-&.

Rook, J. A. F. 1979. Role of Carbohydrate-Metabolism in the Regulation of Milk-Production. Proceedings of the Nutrition Society 38(3):309-314.

Rulquin, H., P. M. Pisulewski, R. Verite, and J. Guinard. 1993. Milk-Production and Composition As A Function of Postruminal Lysine and Methionine Supply - A Nutrient-Response Approach. Livestock Production Science 37(1-2):69-90.

Rulquin, H., S. Rigout, S. Lemosquet, and A. Bach. 2004. Infusion of glucose directs circulating amino acids to the mammary gland in well-fed dairy cows. J. Dairy Sci. 87(2):340-349.

Safayi, S., P. K. Theil, L. Hou, M. Engbaek, J. V. Norgaard, K. Sejrsen, and M. O. Nielsen. 2009. Continuous lactation effects on mammary remodelling during late gestation and lactation in dairy goats. J. Dairy Sci. doi:10.3168/jds.2009-2507.

Sauvant, D., and P. Morand-Fehr. 1989. Goats. Pages 169-180 in Ruminant Nutrition: Recommended Allowances and Feed Tables. R. Jarrige, ed. John Libbey and Co., Ltd., London.

Schei, I., A. Danfaer, I. A. Boman, and H. Volden. 2007. Post-ruminal or intravenous infusions of carbohydrates or amino acids to dairy cows 1. Early lactation. Animal 1(4):501-514.

Scott, R. A., D. E. Bauman, and J. H. Clark. 1976. Cellular Gluconeogenesis by Lactating Bovine Mammary Tissue. J. Dairy Sci. 59(1):50-56.

Seymour, W. M., C. E. Polan, and J. H. Herbein. 1990. Effects of Dietary-Protein Degradability and Casein Or Amino-Acid Infusions on Production and Plasma Amino-Acids in Dairy-Cows. J. Dairy Sci. 73(3):735-748.

Sharma, R., and V. K. Kansal. 2000. Heterogeneity of cationic amino acid transport systems in mouse mammary gland and their regulation by lactogenic hormones. Journal of Dairy Research 67(1):21-30.

Shennan, D. B., and M. Peaker. 2000. Transport of milk constituents by the mammary gland. Physiological Reviews 80(3):925-951.

Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Setala. 1991. A Nordic Proposal for An Energy Corrected Milk (Ecm) Formula. Performance Recording of Animals : State of the Art, 1990 50:156-&.

Strudsholm, F., O. Aaes, J. Madsen, V. F. Kristensen, H. R. Andersen, T. Hvelplund, and S. Oestergaard. 1999. Danske fodernormer till Kvaeg (Danish feed recommendations for cattle). Pages 1-47 in Report No. 84. Landsudvalget for Kvaeg, Aarhus.

Tygesen, M. P., M. O. Nielsen, P. Norgaard, H. Ranvig, A. P. Harrison, and A. H. Tauson. 2008. Late gestational nutrient restriction: Effects on ewes' metabolic and homeorhetic adaptation, consequences for lamb birth weight and lactation performance. Archives of Animal Nutrition 62(1):44-59.

Weekes, T. L., P. H. Luimes, and J. P. Cant. 2006. Responses to amino acid imbalances and deficiencies in lactating dairy cows. J. Dairy Sci. 89(6):2177-2187.

Wilde, C. J., and C. H. Knight. 1989. Metabolic Adaptations in Mammary-Gland During the Declining Phase of Lactation. J. Dairy Sci. 72(6):1679-1692.

Table 1- The chemical composition of the feed used in both early and late lactation.

Nutrient		SFU[1]	AAT[2]	PBV[3]	Fatty acids	Sugar	Starch	Ca	P	Mg
	DM (%)	SFU/kg DM	g/SFU	g/SFU	g/SFU	g/SFU	g/SFU	g/SFU	g/SFU	g/SFU
Barley	85	1.13	84	-36	25	18	517	0.6	3.6	1.1
Hay	93	0.61	135	18	17	112	0	5.0	2.0	2.0
Urea	100	1	0	2880	0	0	0	0	0	0
Protected fat	99	2.35	0	0	310	0	0	30	0	0

[1]One Scandinavian Feed Unit (SFU) = 7890 KJ of net energy/kg of DM, [2]Amino acids absorbed from the small intestine (AAT), [3]Protein balance in rumen (Strudsholm et al., 1999).

Table 2- An overview of the daily provision of energy and protein to the goats by the diet and with infusion solutions for a goat with a body weight of 50 kg and a milk yield of 3 and 1.5 kg per day in early and late lactation, respectively.

*

Daily dietary provision (g/d except otherwise stated)	EL Body weight = 50 kg milk yield = 3 kg/d				LL Body weight = 50 kg milk yield = 1.5 kg/d			
AAT	181.11				136.11			
Hay	400				400			
Barley	1420				970			
Protected fat	70				80			
Urea	16				10			
Vit/Min	20				10			
Dietary NE (MJ/d)	13.93				10.71			
Daily provision via infusion (g/d unless otherwise stated)	EAA	ACE	GLU	SAL	EAA	ACE	GLU	SAL
kJ NE/d	560.9	560.9	560.9	0	421.5	421.5	421.5	0
Osmoles /d*	2.16	2.16	2.16	2.16	1.62	1.62	1.62	1.62
EAA in total	45.28				34.01			
Ile	4.78				3.59			
Leu	8.77				6.59			
Met	2.12				1.59			
Phe	4.18				3.14			
Thr	4.50				3.39			
Trp	1.76				1.31			
Val	6.67				5.01			
Arg	2.72				2.04			
His	2.52				1.90			
Lys	7.26				5.45			
Glucose			53.93				40.53	
Acetic acid (100%)		64.77				48.68		
NaCl	46.35		54.28	63.03	33.33		40.80	47.38
mol ATP/d	10.8	10.8	10.8	0	8.1	8.1	8.1	0

*Omsolarity of infusion solutions were 2.25 in EL and 1.69 in LL

Table 3- Milk yield and its fat and protein contents in early and late lactating dairy goats fed a restricted diet supplemented by intravascular infusion of nutrients.

	Treatment					P-value
	EAA	ACE	GLU	SAL	SEM	T
Milk yield (g/last 48h)						
EL	4821^{ab}	4765^{ab}	5140^{a}	4237^{b}	547	0.06
LL	2888	2732	2787	2861	606	0.71
ECM (g/last 48h)						
EL	5188	5761	5393	4959	602	0.19
LL	3547^{a}	3040^{ab}	2775^{b}	3118^{ab}	602	0.03
Protein (%)						
EL	3.48^{a}	3.46^{a}	3.06^{b}	3.38^{a}	0.15	0.03
LL	3.98^{a}	3.76^{ab}	3.52^{b}	3.74^{ab}	0.14	0.13
Protein yield (g/last 48h)						
EL	166^{a}	163^{ab}	157^{ab}	139^{b}	14	0.11
LL	114^{a}	100^{ab}	98^{b}	106^{ab}	14	0.09
Fat (%)						
EL	4.49^{a}	5.81^{b}	4.61^{a}	5.44^{b}	0.33	0.01
LL	5.51^{a}	5.50^{a}	3.86^{b}	4.51^{ab}	0.32	0.02
Fat yield (g/last 48h)						
EL	222	272	238	232	31	0.26
LL	160^{a}	130^{a}	108^{b}	130^{a}	31	0.04

EAA: essential amino acids, ACE: acetate, GLU: glucose, Sal: saline, T: Treatment, S: Stage of lactation, EL: early lactation, LL: late lactation. Different letters within a row indicate significant difference between treatments.

Table 4- Arterial concentrations (A), arterial -milk vein concentration differences (AVD), and mammary extraction rates (E) for plasma metabolites, IGF-1 and insulin in early and late lactating dairy goats fed a restricted diet supplemented by intravascular infusion of nutrients.

		Treatment					P-value		
		EAA	ACE	GLU	SAL	SEM	T	S	T x S
GLU									
EL									
	A (mM)	4.00^{ac}	3.95^{a}	4.60^{b}	4.22^{c}	0.14	<0.001	0.02	<0.06
	AVD (mM)	1.27^{a}	1.18^{b}	1.24^{ab}	1.21^{ab}	0.16	<0.01	<0.001	<0.01
	E ratio	0.32^{a}	0.30^{ab}	0.28^{b}	0.29^{b}	0.04	<0.001	<0.001	<0.001
LL									
	A (mM)	4.00^{a}	3.99^{a}	4.24^{b}	3.97^{a}	0.15			
	AVD (mM)	1.12^{a}	1.04^{a}	0.88^{b}	1.16^{a}	0.16			
	E ratio	0.27^{a}	0.26^{a}	0.20^{b}	0.29^{a}	0.04			
ACE									
EL									
	A (mM)	0.78^{a}	0.93^{ab}	0.59^{c}	0.64^{ac}	0.08	0.01	0.65	0.54
	AVD (mM)	0.64^{a}	0.75^{b}	0.48^{c}	0.52^{c}	0.05	<0.001	0.22	0.04
	E ratio	0.81	0.79	0.82	0.80	0.02	0.98	<0.001	0.07
LL									
	A (mM)	0.73^{a}	0.91^{ab}	0.61^{ac}	0.79^{a}	0.08			
	AVD (mM)	0.53^{a}	0.72^{b}	0.42^{c}	0.60^{a}	0.05			
	E ratio	0.75^{a}	0.78^{ab}	0.74^{ac}	0.77^{a}	0.02			
BHB									
EL									
	A (mM)	0.41^{a}	0.33^{b}	0.25^{c}	0.48^{d}	0.04	<0.001	<0.001	0.46
	AVD (mM)	0.28^{a}	0.21^{b}	0.17^{c}	0.32^{d}	0.03	<0.001	0.51	<0.01
	E ratio	0.68^{a}	0.64^{b}	0.67^{ab}	0.66^{ab}	0.04	0.01	<0.001	<0.001
LL									
	A (mM)	0.48^{a}	0.47^{a}	0.36^{b}	0.56^{c}	0.04			
	AVD (mM)	0.23^{a}	0.26^{a}	0.15^{b}	0.32^{c}	0.03			
	E ratio	0.51^{a}	0.53^{ac}	0.45^{b}	0.54^{c}	0.04			
NEFA									
EL									
	A (µEq)	392^{a}	454^{a}	309^{b}	544^{c}	35	<0.001	<0.001	<0.001
	AVD (µEq)	157^{a}	155^{a}	111^{b}	238^{c}	18	<0.001	<0.001	<0.001
	E ratio	0.31	0.30	0.30	0.38	0.06	0.17	<0.001	0.66
LL									
	A (µEq)	375^{a}	266^{b}	236^{b}	279^{b}	36			
	AVD (µEq)	126^{a}	57^{b}	72^{b}	81^{b}	19			
	E ratio	0.17	0.15	0.08	0.17	0.06			
TG									
EL									
	A (mM)	0.16	0.16	0.16	0.15	0.02	0.18	<0.001	0.01
	AVD (mM)	0.10	0.10	0.10	0.09	0.02	<0.01	<0.001	<0.001
	E ratio	0.62^{a}	0.64^{a}	0.63^{a}	0.51^{b}	0.04	<0.01	<0.001	<0.001
LL									
	A (mM)	0.23^{a}	0.23^{a}	0.20^{a}	0.27^{c}	0.02			
	AVD (mM)	0.11^{a}	0.13^{ac}	0.09^{b}	0.14^{c}	0.02			

		Treatment					P-value		
		EAA	ACE	GLU	SAL	SEM	T	S	T x S
	E ratio	0.50[a]	0.55[a]	0.42[b]	0.51[a]	0.04			
LCFA									
EL									
	A (µEq)	487	481	483	465	66	0.18	<0.001	0.06
	AVD (µEq)	307	309	305	268	58	<0.01	<0.001	<0.001
	E ratio	0.62[a]	0.64[a]	0.63[a]	0.51[b]	0.04	<0.01	<0.001	<0.001
LL									
	A (µEq)	680[a]	688[a]	617[a]	817[b]	67			
	AVD (µEq)	330[a]	376[a]	262[b]	412[a]	59			
	E ratio	0.50[a]	0.55[a]	0.42[b]	0.51[a]	0.04			
Urea									
EL	A (mM)	2.65[a]	3.36[b]	2.34[a]	2.60[a]	0.50	<0.01	<0.001	0.20
LL	A (mM)	4.70[a]	4.54[ab]	4.01[b]	3.97[b]	0.50			
IGF1							0.64	0.74	0.98
EL	A (nM)	55.0	46.9	53.3	46.9	8.3			
LL	A (nM)	53.3	49.2	56.2	49.7	8.3			
Insulin							0.30	0.55	0.66
EL	A (µg/l)	0.60	0.43	0.40	0.43	0.12			
LL	A (µg/l)	0.59	0.40	0.58	0.45	0.12			

T: Treatment, S: Stage of lactation, EL: early lactation, LL: late lactation, EAA: essential amino acids, ACE: acetate, GLU: glucose, Sal: saline, BHB: beta hydroxyl butyrate, NEFA: non-esterified fatty acids, TG: triglyceride, LCFA: long chain fatty acids, IGF1: insulin growth factor 1, A: arterial concentration, AVD: arterial-milk vein difference, E ratio: extraction rate. Different letters within a row indicate significant difference between treatments.

Table 5- Arterial concentrations (A), arterial -milk vein concentration differences (AVD), and mammary extraction rates (E) for blood acid-base parameters in early and late lactating dairy goats fed a restricted diet supplemented by intravascular infusion of nutrients.

		Treatment					P-value		
		EAA	ACE	GLU	SAL	SEM	T	S	T x S
TO2									
EL									
	A (mM)	5.38	5.52	5.39	5.33	0.22	0.64	0.02	0.26
	AVD (mM)	2.77^{a}	2.57^{ac}	2.25^{bc}	2.37^{c}	0.17	<0.001	0.19	<0.001
	E ratio	0.52^{a}	0.47^{b}	0.42^{c}	0.44^{bc}	0.03	<0.001	0.47	<0.001
LL									
	A (mM)	5.53^{a}	5.57^{ab}	5.52^{a}	5.83^{b}	0.22			
	AVD (mM)	2.53^{a}	2.41^{ab}	2.24^{b}	3.09^{c}	0.18			
	E ratio	0.46^{a}	0.43^{ab}	0.40^{b}	0.54^{c}	0.03			
TCO2									
EL									
	A (mM)	24.0^{a}	39.6^{b}	25.9^{c}	23.9^{a}	0.98	<0.001	0.31	0.03
	AVD (mM)	-2.41^{a}	-2.27^{a}	-1.87^{b}	-2.47^{a}	0.30	<0.001	<0.001	0.20
	E ratio	-0.10^{a}	-0.05^{b}	-0.08^{c}	-0.11^{a}	0.01	<0.001	<0.01	0.17
LL									
	A (mM)	26.0^{a}	37.7^{b}	26.7^{ac}	25.0^{ad}	0.99			
	AVD (mM)	-2.62^{a}	-2.91^{b}	-2.17^{c}	-3.22^{ab}	0.31			
	E ratio	-0.10^{a}	-0.08^{b}	-0.08^{b}	-0.13^{c}	0.01			
Mammary RQ							0.50	0.09	0.79
EL		0.87	0.93	0.90	0.94	0.09			
LL		0.97	1.17	0.96	0.99	0.09			
HCO_3									
EL									
	A (mM)	22.9^{a}	38.3^{b}	24.8^{c}	22.8^{a}	0.95	<0.001	0.36	0.03
	AVD (mM)	-2.19^{a}	-2.14^{a}	-1.67^{b}	-2.33^{a}	0.29	<0.001	<0.001	0.40
	E ratio	-0.10^{a}	-0.05^{b}	-0.07^{c}	-0.11^{a}	0.01	<0.001	<0.01	0.38
LL									
	A (mM)	24.8^{a}	36.3^{b}	25.5^{ac}	23.8^{ad}	0.97			
	AVD (mM)	-2.39^{ac}	-2.71^{a}	-1.96^{b}	-2.94^{ad}	0.30			
	E ratio	-0.10^{a}	-0.07^{b}	-0.08^{b}	-0.13^{c}	0.01			
pH									
EL	A	7.41^{a}	7.53^{b}	7.45^{c}	7.44^{c}	0.01	<0.001	<0.01	0.06
LL	A	7.41^{a}	7.51^{b}	7.43^{c}	7.41^{a}	0.01			

T: Treatment, S: Stage of lactation, EL: early lactation, LL: late lactation, EAA: essential amino acids, ACE: acetate, GLU: glucose, Sal: saline, TCO2: total CO_2, TO2: total O_2, RQ: respiratory quote, A: arterial concentration, AVD: arterial-milk vein difference, E ratio: extraction rate. Different letters within a row indicate significant difference between treatments.

Activities in relation to PhD education
(since September 2004)

Papers and presentations

- **Safayi, S.**, and M. O. Nielsen. Intravenous Supplementation of Acetate, Glucose or Essential Amino Acids to an Energy and Protein Deficient Diet in Early and Late Lactating Dairy Goats: Effects on Milk Production and Mammary Nutrient Extraction. *(to be submitted to J. Dairy Sci.)*
- **Safayi, S.**, P. K. Theil, V. S. Elbrønd, L. Hou, M. Engbaek, J. V. Norgaard, K. Sejrsen, and M. O. Nielsen. 2009. Mammary Remodelling in Primiparous and Multiparous Dairy Goats During Lactation. J. Dairy Sci. *(Accepted; JDS-09-2422.R2)*
- **Safayi, S.**, P. K. Theil, L. Hou, M. Engbaek, J. V. Norgaard, K. Sejrsen, and M. O. Nielsen. 2009. Continuous Lactation Effects on Mammary Remodelling During Late Gestation and Lactation in Dairy Goats. J. Dairy Sci. *(In press; doi:10.3168/jds.2009-2507)*
- **Safayi, S.**, and M. O. Nielsen. 2009. Continuous lactation effects on mammary extraction rates of nutrients in dairy goats. Pages 652-653 in The XIth International Symposium on Ruminant Physiology (ISRP). Vol. Clermont-Ferrand, France. Y. Chilliard, F. Glasser, Y. Faulconnier, F. Bocquier, I. Veissier, and M. Doreau, eds. Wageningen Academic Publishers, Wageningen, The Netherlands.
- Nørgaard J. V., M. O. Nielsen, P. K. Theil, M. T. Sørensen, **S. Safayi**, and K. Sejrsen. 2008. Development of mammary gland of fat sheep submitted to restricted feeding during late pregnancy. Small Ruminant Research. 76:155-165.
- **Safayi, S.**, P. K. Theil, V. S. Elbrønd, L. Hou, M. Engbaek, and M. O. Nielsen. 2008. Mammary development and remodelling in primiparous and multiparous dairy goats during lactation, CRU Symposium on 'Lactation in Mammals and Humans', Uppsala, Sweden. *(Oral presentation)*
- **Safayi, S.**, P. K. Theil, V. S. Elbrønd, L. Hou, M. Engbaek, and M. O. Nielsen. 2008. Effect of continuous lactation in dairy goats on mammary histology, cell turnover and expression of angiogenic factors, The 5th European Gordon Research Conference on Mammary Gland Biology, Lucca (Barga), Italy. *(Poster presentation)*
- **Safayi, S.**, P. K. Theil, and M. O. Nielsen. 2007. Effect of parity and continuous lactation on expression of angiogenic factors during different stages of lactation in the mammary gland of dairy goats, The 1st SHARE (Synergy in Human and Animal Research) Symposium, Copenhagen, Denmark. *(Poster presentation)*

Other publications

- Bolliger A. M., Muguerza N. B., Grossi A., **Safayi S.** 2006. The PhD Students' Network at KVL –Is It Dying Out?. Det Levende Universitet (KVL's Magazine), 1:22-23.
- Gjerstad R. T., Nielsen D., Andren M., Holso K., Lindberg H., Solli M., Babigumira R., Bjonnes I., Brydolf S., Areskog M., Horn I., Jor E., Lundberg D., Vuorinen A., **Safayi S.** 2005. The Students' Perspective: NOVA Student Forum, in book "10 Years NOVA-UN 1995-2005". 118:119.
- **Safayi S**. 2005. KVL – An International University, Det Levende Universitet (KVL's Magazine), 6:7.

Research projects

2009 Participant in project on *"Role of Prostaglandin E2 (PGE2) on Growth and Differentiation of Bovine Mammary Epithelial Cells"* (with University of Arizona, AZ, USA)

2007-2009 Participant in project on "*Continuous lactation in dairy cows"* (with Swedish Agricultural University, Sweden)

2006-2009 PhD student in project on "*Continuous lactation and dry period feeding: Impact on pre- partum mammary redevelopment and milk production potential in ruminants"*

2006 Participant in project on "*Starch for dairy cows"* (with Aarhus University, Denmark)

2004-2006 PhD student in project on "*Role of mammary epithelial cell turnover and nutrient supply in regulation of milk production in small ruminants"*

PhD courses (60.5 ECTS + 1 audit course)

2009 *¨Medical Scientific English¨* (2.5 ECTS), Faculty of Health Sciences, University of Aarhus, Denmark

2007-2008 *¨Applies Statistics¨*, Faculty of Life Sciences, University of Copenhagen, Denmark *(audit course)*

2007 *¨Write Publishable Research: An Interactive Process¨* (2.5 ECTS), Copenhagen Business School, Denmark

¨Stereology: Sampling and Measurement of 3D Structures¨ (3 ECTS), Faculty of Life Sciences, University of Copenhagen, Denmark

¨Image Analysis¨ (7.5 ECTS), Faculty of Life Sciences, University of Copenhagen, Denmark

¨Real Time RT-PCR Advanced¨ (4 ECTS), Faculty of Science, University of Copenhagen, Denmark

¨Genome & Cell Biology¨ (12 ECTS), Faculty of Life Sciences, University of Copenhagen, Denmark

2005 *¨Real Time PCR¨* (2.5 ECTS), TATAA Biocenter, Dept. of Molecular Biotechnology, Chalmers University of Technology, Gothenburg, Sweden

¨Laboratory Animal Science¨ Category C (9 ECTS), Faculty of Life Sciences, University of Copenhagen, Denmark

¨The R System for Computational Data Analysis¨ (2 ECTS), Danish Informatics Network in the Agricultural Sciences (DINA) Research School, Denmark

¨Statistics for Veterinarians¨ (9 ECTS), Faculty of Life Sciences, University of Copenhagen, Denmark

2004 *¨Immunodiagnostic Methods for Veterinarians¨* (6 ECTS), Faculty of Life Sciences, University of Copenhagen, Denmark

Teaching experiences

2007-2008 Co-supervising two MSc thesis' projects on "*Lactation Physiology"*, Faculty of Life Sciences, University of Copenhagen, Denmark

2005-2008 *¨Experimental animal nutrition and physiology¨* postgraduate courses (theoretical and practical parts), Faculty of Life Sciences, University of Copenhagen, Denmark

2007 *¨Veterinary physiology¨* undergraduate course, Faculty of Life Sciences, University of Copenhagen, Denmark

Workshops

2005 II International ¨COST B20 Action¨ Workshop on *¨Mammary Gland Development, Function and Cancer: Proteins and signaling pathways controlling mammary epithelial cell function¨*, Institute for Biochemical Research, Georg-Speyer-Haus, Frankfurt, Germany

¨Scientific Information Sources and Information Management¨ Workshop, Faculty of Life Sciences, University of Copenhagen, Denmark

¨Databases and Web Programming¨ Workshop, Danish Informatics Network in the Agricultural Sciences (DINA), Tune Landboskole, Roskilde, Denmark

Board memberships

2006- 2008 PhD Study Committee, Graduate School, Faculty of Life Sciences, University of Copenhagen, Denmark

2005-2007 The Students' Forum Board (NSF) and Local NOVA-UN Team, The Nordic Forestry, Veterinary and Agricultural University Network (NOVA-UN), Nordic Countries

2004-2007 PhD Network, Faculty of Life Sciences, University of Copenhagen, Denmark

2004-2006 Iranian Academic Society in Denmark (IASD), Denmark